Dissertation

The regulatory network adjusting light-harvesting in the model green alga *Chlamydomonas reinhardtii*

zur Erlangung des akademischen Grades
Doktor der Naturwissenschaften (Dr. rer. nat.)

vorgelegt von
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April 2015

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Zusammenfassung

In photosynthetischen Organsimen ist die Regulation der Lichtsammlung eine Schlüsselkomponente zur effizienten Umsetzung von Licht und anorganischem Kohlenstoff in Biomasse. In dieser Dissertation wurde das Zusammenspiel von Kurz- und Langzeitmechanismen untersucht, die die Lichtsammelantenne am Photosystem II (PSII) im Modellorganismus *Chlamydomonas reinhardtii* steuern. Diese einzellige Grünalge setzt Energie und Kohlenstoff neben photosynthetischer CO_2-Fixierung auch heterotroph um. Ein Mangel an anorganischem oder die Verfügbarkeit von organischem Kohlenstoff führt zu einer übermäßigen Reduktion der photosynthetischen Elektronentransportkette und damit zur Überanregung des PSII. Durch Kombination von molekularbiologischen und biochemischen Methoden mit physiologischen und Chlorophyllfluoreszenzanalysen konnte in dieser Arbeit gezeigt werden, dass eine Abnahme der funktionalen Antennengröße den Anregungsdruck auf PSII unter CO_2-Mangel effizient senkte. Insbesondere wurde deutlich, dass Translationskontrolle der Hauptlichtsammelproteine (LHCII) protektive Kurzzeitmechanismen wie LHCII *state transtitions* langfristig ersetzt.

Der LHCII Translationsrepressor NAB1 stellte dabei einen zentralen Faktor für die Anpassung an ein verändertes Kohlenstoffangebot dar. Durch eine verstärkte Aktivität des nuklearen *NAB1*-Promoters wurde der Repressor unter CO_2-Mangel verstärkt exprimiert. Ein veränderter Gehalt an NAB1 in einer *state transition* Mutante deutete zusammen mit einer ausbleibenden NAB1-Akkumulation nach Inhibition der Photosynthese darauf hin, dass plastidäre Signale die *NAB1*-Transkription im Kern steuern. Um die Promoterelemente am Ende des retrograden Signalweges zu identifizieren wurde ein Reporterkonstrukt für Promoteranalysen entwickelt. Systematische Deletionsstudien und Datenbankrecherchen resultierten im Auffinden eines Promoterfragments von 152 Basen, das bisher unbekannte *cis*-regulatorische Elemente kodiert.

Weiter konnte gezeigt werden, dass moderate Veränderungen der Lichtintensität die LHCII Translationsrepression im Zytosol durch Redox-Kontrolle von NAB1 regulieren. Spezifische Nitrosylierung des Cysteinrests 226 und Reduktion durch Thioredoxin h1 passte die NAB1-Aktivität dem Bedarf an Lichtsammelproteinen an. Dies ist die erste Beschreibung eines Redox-Mechanismus, der die Synthese von kernkodierten Proteinen der Photosynthese steuert.

Insgesamt wurden in dieser Arbeit regulatorische Vernetzungen aufgedeckt, die die Lichtsammlung über verschiedene Zeiträume der Verfügbarkeit von Licht und Kohlenstoff anpassen sowie die Expressionskontrolle in Kern und Zytosol mit Kurzzeitmechanismen im Chloroplasten verbinden. Es wurden so neue Einblicke in die intrazelluläre Kommunikation gewonnen, die eine optimale Absorption und Umsetzung der Lichtenergie sicherstellt.

Summary

In photosynthetic organisms, control of light-harvesting is a key component of acclimation mechanisms that optimize photon conversion efficiencies. In this thesis, the interrelation of short- and long-term regulation of light-harvesting at photosystem II (PSII) was analyzed in the green alga *Chlamydomonas reinhardtii*. This model organism is able to gain carbon and energy through photosynthetic carbon dioxide fixation as well as heterotrophic feeding. A lowered inorganic or increased organic carbon supply reduces the rate of NADPH consumption by the Calvin cycle, resulting in an over-reduced photosynthetic electron transport chain and increased excitation pressure at photosystem II. A combination of molecular biology, biochemistry, chlorophyll fluorescence and physiological analyses revealed that a reduction in functional antenna size efficiently relieved excitation pressure on PSII under these conditions. Particularly, translation control on PSII-associated major light-harvesting proteins (LHCII) replaced state transitions as an initial protection mechanism in the long term.

The LHCII translation repressor NAB1 emerged as key factor implicated in the acclimation to the prevailing carbon assimilation mode. The level of NAB1 was increased under carbon dioxide limitation, and expression control based on modulated promoter activity. Application of a photosynthetic electron transport inhibitor and a perturbed NAB1 accumulation in a state transition mutant suggested that chloroplast retrograde signals control nuclear *NAB1* expression. To further investigate this retrograde signaling, a reporter system was developed that enables detailed promoter analyses. Systematic truncation studies identified a promoter fragment of 152 bases, which comprised essential regulatory elements and can be used as tool for the identification of *cis*-regulatory elements in future studies.

Furthermore, chloroplast redox poise was shown to modulate the extent of LHCII translation repression in the cytosol via cysteine based redox control of NAB1. In response to moderate light intensity changes, a fine-tuning system comprising specific single cysteine nitrosylation and thioredoxin mediated re-reduction adjusted NAB1 activity to the demand for light-harvesting antenna proteins. This is the first mechanistic description of redox based translation control of nuclear encoded photosynthesis associated genes.

Overall, this thesis describes regulatory circuits that adjust light-harvesting capacity over a range of time scales, involving nuclear and cytosolic expression control as well as short-term responses in the chloroplast, and provides new insights into interorganellar communication that ensures optimal photon capture.

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Abbreviations

ASC	ascorbate
ATP, ADP, AMP	adenosine tri-, di-, monophosphate
A. thaliana	*Arabidopsis thaliana*
bp	base pair(s)
C181S, C226S	replacements of cysteine with serine at position 181 or 226
CAH1	carbonic anhydrase 1
*c*CA	secretion signal of *C. reinhardtii* CAH1
CCM	carbon concentrating mechanism
CEF	cyclic electron flow
CMC	carboxy-methyl cellulose
CO_2	carbon dioxide
CoA	coenzyme A
CP	monomeric, minor light-harvesting antenna complex
cPTIO	2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
CRE	*cis*-acting regulatory element
CSD	cold shock domain
cyt b_6f or cyt *c*	cytochrome b_6f or cytochrome *c* complex
C. reinhardtii	*Chlamydomonas reinhardtii*
Da	Dalton
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DEA-NONOate	1,1-Diethyl-2-hydroxy-2-nitroso-hydrazine sodium salt
DTT	dithiothreitol
ECS	electrochromic shift
et al.	and others
Fd	ferredoxin
FNR	ferredoxin $NADP^+$ reductase
GAP	glyceraldehyde-3-phosphate
GAR	glycine arginine rich
*g*Luc	*Gaussia princeps* luciferase
GSH, GSSG	glutathione (c-L-glutamyl-L-cysteinylglycine), reduced or oxidized
GSNO(R)	S-nitrosoglutathione (reductase)
H_2O_2	hydrogen peroxide
HA-tag	protein tag derived from human influenza hemagglutinin
HSM	high salt media
HSP70A	heat shock protein 70 version A
IB	immunoblot
k.o.	knock out
LEF	linear electron flow
LHCI, LHCII	major light-harvesting complex of photosystem I and II

LHCBM1 to LHCBM9	isoforms of the major light-harvesting antenna of photosystem II
LHCSR3	stress related LHC 3
*Lp*IBP	*Lollium perenne* ice binding protein
M	mol/L
MAL	malate
NAB1	nucleic acid binding protein 1
$NAD(P)^+$, NAD(P)H	nicotinamide adenine dinucleotide (phosphate), oxidized or reduced
NaR	nitrate reductase
NO(S)	nitric oxide (synthase)
NPQ	non-photochemical quenching of chlorophyll fluorescence
NTR	NADPH dependent thioredoxin reductase
$^1O_2$	singlet oxygen
OAA	oxaloacetate
oex	over-expression
Pc	plastocyanin
PCR	polymerase chain reaction
PET	photosynthetic electron transport
ΦPSII	photosystem II quantum yield in the light
PSI or PSII	photosystem I or II
PSBS	a four helix LHC relative
PTM	posttranslational modification
PQ, PQH_2	plastoquinone, plastoquinole
qE	energy dependent quenching of chlorophyll fluorescence
qI	photoinhibition
qP	photochemical quenching of chlorophyll fluorescence
qT	state transitions
RLU	relative luminescence units
ROS	reactive oxygen species
RP	recombinant protein
RRM	RNA recognition motif
qRT-PCR	quantitative real-time reverse transcription PCR
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH, SNO, SX	cysteine residue in thiol, nitrosylated or unspecified state
STT7	state transition thylakoid 7 kinase in *Chlamydomonas reinhardtii*
TAP	tris acetate phosphate
TATA-box	thymine-adenine-thymine-adenine nucleotide sequence motif
TPT	triose phosphate translocator
TRX	thioredoxin
TSP	total soluble protein
TSS	transcription start site
UTR	untranslated region
wt	wild-type

1 Introduction

Photosynthesis is the base of the food chain and provides energy used in form of fuels. As fossil energy sources and agricultural area become limiting, there is increasing interest to enhance the utilization of light energy both by cellular and cell-free systems. All approaches have in common that their value directly depends on efficient photon capture and conversion. In photosynthetic organisms light-harvesting systems evolved to efficiently absorb light and transfer excitation energy (Büchel, 2015; Ruban, 2015). Control of the light-harvesting antenna is therefore a key process optimizing photon conversion efficiencies.

In this thesis, molecular mechanisms behind the regulation of light-harvesting in *Chlamydomonas reinhardtii* were investigated. This unicellular green alga is an excellent model organism for the analysis of photosynthesis, amongst others because of the ease of cultivation and manipulation, sequenced genomes, an expanding molecular toolbox and the viability of photosynthetic mutants (Elrad and Grossman, 2004; Merchant et al., 2007). Many findings are of relevance for research on higher plants as well.

However as an 'acetate flagellate' (Harris, 2009), *C. reinhardtii* comprises key characteristics from both the plant and animal kingdoms. The green alga is unicellular with frequent cell divisions, motile and able to acquire light and carbon from both photosynthetic fixation of carbon dioxide and feeding on organic carbon (Figure 1-1). *C. reinhardtii* harbors one cup-shaped chloroplast, in contrast to higher plants cells with many, presumably heterogeneous plastids (Lepistö et al., 2012). Regulation of light-harvesting, signaling and acclimation responses obviously vary from that of higher plants. In particular, the coordination of short- and long-term responses adjusting light-harvesting in *C. reinhardtii* is in the center of interest within this thesis.

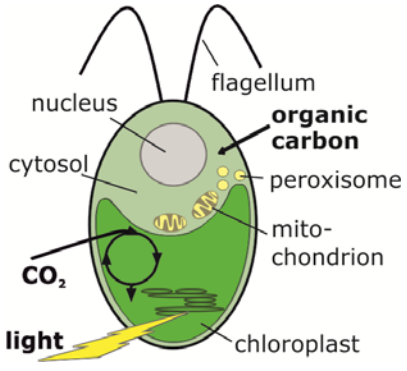

Figure 1-1 The unicellular green alga *Chlamydomonas reinhardtii*.
Scheme of a vegetative cell with two flagella, one nucleus, peroxisomes and a reticular network of mitochondria around the single cup-shaped chloroplast. Other organelles are omitted for reasons of clarity. Major light and carbon acquisition pathways are indicated (bold) and depicted in Figure 1-2.

This chapter (1) introduces mechanisms behind light and carbon acquisition (1.1), regulation of light harvesting (1.2) and signaling networks (1.3), before the specific aims of this thesis are defined (chapter 2).

Chapter 3 describes the development of a versatile reporter system that enables the analysis of nuclear promoters utilizing efficient secretion of reporter proteins into the culture medium.

This system was subsequently applied in combination with molecular biology, biochemistry, chlorophyll fluorescence and physiological analyses to analyze the multi-compartmental short- and long-term regulation of light-harvesting under varying carbon supply in chapter 4.

Chapter 5 elucidates details of the mechanism behind redox based control of light-harvesting protein synthesis under fluctuating light intensities involving chloroplast to cytosol signaling pathways.

The results shown in chapters 3 to 5 were obtained in collaboration, and sections 3.2 and 4.2 were published as peer-reviewed journal articles. The authors' contributions are described in the respective sections.

In chapter 6, the novel findings are critically discussed against the background of retrograde signaling networks and photosynthetic acclimation responses, and incorporated into a model depicting strategies to regulate light-harvesting at photosystem II under varying light and carbon supply.

Chapter 7 compiles main conclusions of this thesis answering the questions raised, and provides perspectives for future research.

1.1 Energy and carbon acquisition in *Chlamydomonas reinhardtii*

The metabolism of *C. reinhardtii* comprises a broad spectrum of pathways to gain and partition energy and carbon (Johnson and Alric, 2013). The work presented here focuses on pathways operating under light and aerobic conditions.

1.1.1 Electron transport systems convert energy

Oxygenic photosynthesis is the process of converting light into chemically bound energy by reducing carbon dioxide to carbohydrates with electrons extracted from water (Ruban, 2015). The photosynthetic light reaction involves four sequentially linked complexes embedded into the thylakoid membrane in the chloroplast (Figure 1-2); photosystems (PS) I and II, cytochrome b_6f complex (cyt b_6f) and adenosine triphosphate (ATP) synthase (ATPase), connected by the mobile carriers plastoquinone/plastoquinole (PQ/PQH_2) and plastocyanin (Pc).

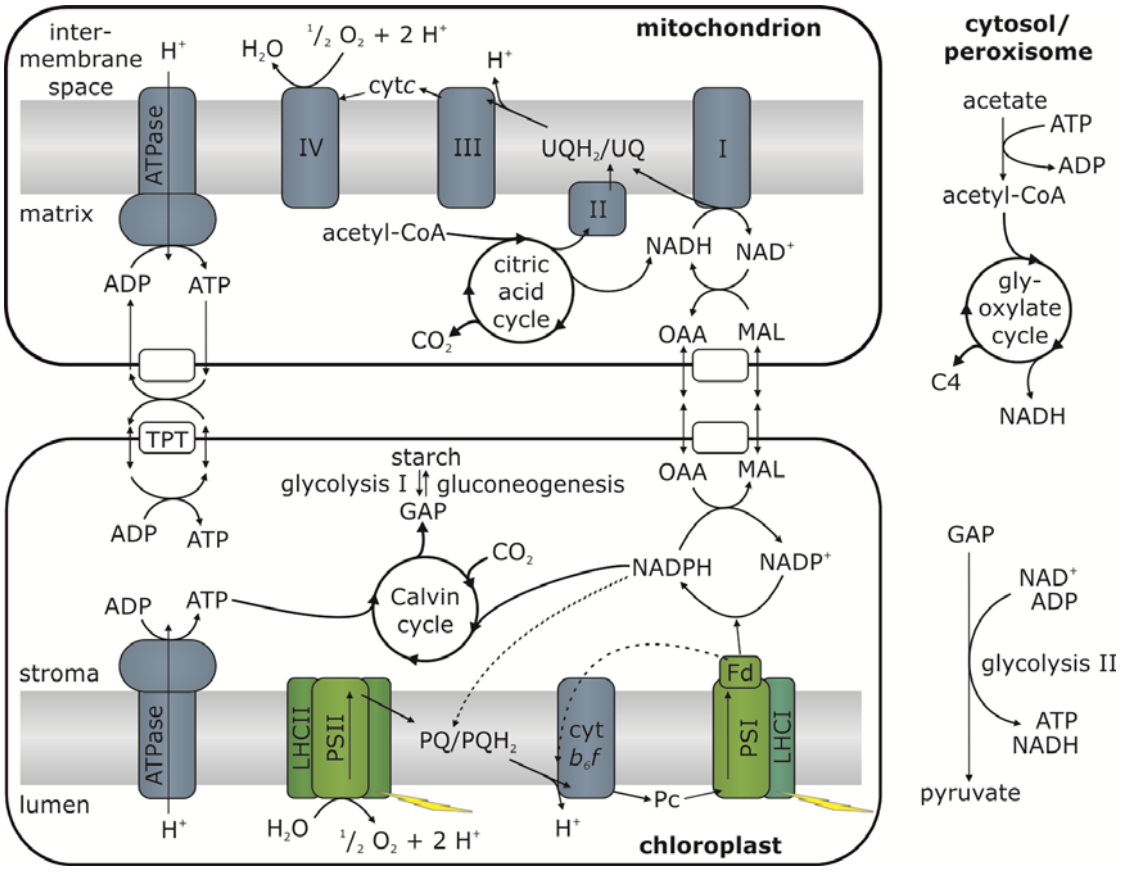

Figure 1-2 Central energy and carbon metabolism in *C. reinhardtii*.
Major metabolic pathways and exchange of energy and carbon between chloroplast, mitochondria, peroxisomes and cytosol; redrawn and extended from Dang et al. (2014). Photosynthesis, gluconeogenesis, glycolysis, respiration and acetate assimilation are outlined (1.1).

Initially, pigments in the light-harvesting complexes around PSII and I (LHCII and LHCI) get excited by photons (Figure 1-2, yellow lightning) and transfer the excitation to the reaction center (Croce and van Amerongen, 2014). Here, the energy is trapped through excitation of the central,

oxidizable chlorophyll, which donates an electron into the photosynthetic electron transport (PET) chain. Downstream of PSI, excited electrons are used to reduce $NADP^+$ (nicotinamide adenine dinucleotide phosphate) to NADPH. Due to water splitting, proton pumping and reduction of $NADP^+$ in the course of electron transport, a pH gradient and electric potential is built up, with the lumen acidified and positively charged. ATPases convert this proton motif force into chemically bound energy by releasing protons into the stroma and using the energy to generate ATP through phosphorylation of adenosine diphosphate (ADP).

NADPH and ATP, the first forms of chemically stable stored light energy, enter the Calvin cycle in the chloroplast stroma, where they are used to reduce carbon dioxide to carbohydrates (1.1.2). To generate one triose phosphate from three CO_2 molecules, nine ATP and six NADPH are required, thus in a ratio of 1.5 : 1 (Lucker and Kramer, 2013). However, linear photosynthetic electron transport from PSII via cyt b_6f to PSI (Figure 1-2) generates these energetic molecules in a ratio of 1.3 : 1. To meet the energetic demands of the cell, cyclic electron flow re-shuttles excited electrons from PSI to plastoquinone and cyt b_6f (Figure 1-2, dashed arrows), therewith pumping additional protons into the lumen and enhancing ATP output (Alric et al., 2010; Iwai et al., 2010a; Alric, 2014; Johnson et al., 2014). The increased pH gradient may also support the induction of energy dependent quenching to prevent photoinhibition (1.2.2). The ratio between linear and cyclic electron transport is fine-tuned, involving complex feedback control (Johnson and Alric, 2012), and in particular LHCII are key components of such photosynthetic acclimation (Bulté et al., 1990; Finazzi et al., 2002; Lucker and Kramer, 2013; 1.2.2).

Excess reducing equivalents can be released into the cytosol and imported into mitochondria via shuttles like the malate/oxaloacetate transport system (Figure 1-2, MAL, OAA) working in conjunction with external NAD(P)H dehydrogenases (Hoefnagel et al., 1998). The mitochondrial electron transport chain, comprised of four complexes (Figure 1-2, I-IV) and the mobile carriers ubiquinone/ubiquinole (UQ/UQH_2) and cytochrome *c* (cyt *c*), converts the reducing power from carbon dissimilation (Figure 1-2, citric acid cycle; 1.1.2) and imported equivalents into a proton motif force. The subsequently generated ATP can be exported to the cytosol via ADP/ATP translocators and to the chloroplast through triose phosphate shuttles (Figure 1-2, TPT; Boschetti and Schmid, 1998; Hoefnagel et al., 1998). Mito-respiratory electron transport therewith substantially contributes to the balance of the energetic molecules NAD(P)H and ATP, and its activity is tightly linked to electron transport in the chloroplast and carbon metabolism (Cardol et al., 2009; Dang et al., 2014).

1.1.2 Carbon metabolism impacts cellular energetics

C. reinhardtii is capable of accumulating biomass through fixation of inorganic carbon, heterotrophic feeding on organic carbon such as acetate and cellulose, or both simultaneously (Harris, 2009; Blifernez-Klassen et al., 2012). Photoautotrophic carbon dioxide assimilation occurs in the Calvin cycle in the chloroplast stroma, with RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) catalyzing the first carbon fixation step. The subsequent reduction to glyceraldehyde-3-phosphate (Figure 1-2, GAP) and regeneration of CO_2 acceptor molecules exploits ATP and NADPH generated in the photosynthetic light reaction (1.1.1). GAP can be converted into any other carbon backbone that is required for cell structure, metabolism or as storage molecule (Johnson and Alric, 2013).

Starch is the preferred storage compound under nutrient replete conditions (Johnson and Alric, 2013). If carbon and energy supply is low, starch is hydrolyzed and metabolized in order to mobilize the reserves. Interestingly, glycolysis is compartmentalized in *C. reinhardtii* (Figure 1-2, glycolysis I and II). The ATP consuming part from glucose to GAP occurs exclusively in the chloroplast, while the ATP releasing generation of pyruvate occurs in the cytosol, accounting for a net transport of energetic molecules (Johnson and Alric, 2012; 1.1.1). In both compartments, the intermediate oxidation of GAP to 3-phosphoglycerate is catalyzed, which releases both NADH and ATP. The final steps of carbon dissimilation take place in the mitochondria. The citric acid cycle breaks down acetyl-CoA into two carbon dioxide molecules, extracting the chemical energy as ATP and reducing equivalents (Figure 1-2). The latter fuel oxidative phosphorylation via mitochondrial electron transport (1.1.1).

During heterotrophic growth, acetate is efficiently taken up from surrounding media and used as a source of carbon and energy (Harris, 2009). In the algal cell, it is converted into acetyl-CoA through ATP consuming reactions (Figure 1-2); either a direct conversion via acetyl-CoA synthetase or a two-step reaction involving acetate kinase and phosphate acetyltransferase (Spalding, 2009). Acetyl-CoA might be dissimilated in the citric acid cycle or, prevalent under non-stressful conditions, assimilated in glyoxylate cycle (Figure 1-2). In this NADH generating cycle, two acetyl-CoA are converted into succinate (Figure 1-2, C4) which in turn might enter the citric acid cycle (see above) or gluconeogenesis (Johnson and Alric, 2013). In higher plants, the glyoxylate cycle occurs in specialized peroxisomes, the glyoxysomes, but in *C. reinhardtii*, the localization of the enzymes is only partly revealed (Hayashi and Shinozaki, 2012; Hayashi et al., 2014).

The prevailing mode of carbon metabolism influences photosynthetic electron transport. On the one hand, inorganic carbon and acetate directly interact with PSII, influencing water oxidation

and its susceptibility to photoinhibition (Shevela et al., 2007; Roach et al., 2013). On the other hand, metabolites might directly serve as signaling molecules, altering expression of photosynthetic genes in the chloroplast and nucleus (Humby et al., 2009; Dietz, 2015; 1.3).

Most important for the work presented here, carbon assimilation and partitioning depends on the availability of reducing equivalents and ATP, and *vice versa* carbon availability influences the cellular redox poise (Geigenberger and Fernie, 2014). The biochemistry of carbon fixation is the limiting step in photosynthesis (Ruban, 2015), and low carbon dioxide availability leads to an insufficient regeneration of $NADP^+$ through the decelerated Calvin cycle. As electron acceptors are limiting, the PET chain becomes over-reduced, measurable as a reduced PQ acceptor site (Dietz et al., 1985) and a decreased photosynthetic quantum yield (Palmqvist et al., 1990; Falk and Palmqvist, 1992; Iwai et al., 2007).

In addition to an accumulation of reducing equivalents, carbon dioxide limitation increases the demand for ATP. Below the CO_2 compensation point, RuBisCO catalyzed oxygenation exceeds the rate of carboxylation, therewith activating energy consuming photorespiration (Wingler et al., 2000). In *C. reinhardtii*, photorespiration is assumed to be essential but its activity is deemed rather low, as the algal cells operate an efficient carbon concentration mechanism that locally increases carbon dioxide concentration around RuBisCO (Moroney et al., 2013). This mechanism comprises a system of carbonic anhydrases and active transporters (Winck et al., 2013b; Wang et al., 2015), which in turn increases the demand for ATP under low CO_2 conditions.

Acetate assimilation consumes ATP as well (see above), and therefore relies on oxidative and photophosphorylation based on enhanced respiration and cyclic electron transport (1.1.1) (Wiessner, 1965). The glyoxylate cycle releases NADH, leading to an excess of reduction equivalents if the respiratory chain is saturated.

Taken together, *C. reinhardtii* uses both inorganic and organic carbon sources and is able to deal with varying carbon dioxide supply. Under photoautotrophic growth with saturating CO_2, linear electron flow is predominant and provides reducing power and ATP for the Calvin cycle. Under carbon dioxide limitation, the PET chain becomes over-reduced and active carbon concentration mechanisms increase the demand for ATP, an imbalance that is even enhanced through simultaneous acetate assimilation. The chloroplast encounters this imbalance with increased photophosphorylation through cyclic electron flow around PSI (1.1.1) and decreased excitation of PSII. LHCII are key components of such photosynthetic acclimation responses, and several regulatory mechanisms operate rapidly (1.2.2) and in the long term (1.2.3) to optimize light-harvesting capacities.

1.2 Light-harvesting and protection of photosystem II

In all photosynthetic organisms light-harvesting initiates the photosynthetic light-energy transformation (1.2.1). The rate of photosynthesis increases with light intensity until the maximum photosynthetic capacity is reached. If the energy absorbed exceeds the photosynthetic capacity, excitation pressure might become harmful and lead to oxidative stress (Barber and Andersson, 1992). Reasons for such an over-excitation are a sudden rise in light intensity as well as a slowdown of downstream processes, for example by substrate limitation (Iwai et al., 2007; 1.1.2; 1.3) or cold temperatures (Huner et al., 1998; Ensminger et al., 2006).

As a motile algae, *C. reinhardtii* cells adjust their positioning through oriented photo-tactic responses to find the optimal light intensity (Feinleib and Curry, 1971). On the molecular level, enhanced excitation of photosystems induces various responses, ranging from altered cellular metabolism (Davis et al., 2013) and dissipation of extra reducing energy through oxygen reduction via alternative oxidases or water-water cycle reaction (Mehler, 1951; Asada, 2000; Cournac et al., 2002), to detoxification and repair mechanisms (Murik et al., 2014; Miret and Munné-Bosch, 2015).

As a fundamental response, light-energy input is balanced through optimizing harvesting capacity (Anderson et al., 1995). Various strategies evolved to balance energy supply, including a broad diversity in structure, function and regulation of light-harvesting complexes (Büchel, 2015; Ruban, 2015). In the following, current knowledge about the photosynthetic machinery of the green lineage is compiled, with emphasis on light-harvesting (1.2.1) and protection (1.2.2; 1.2.3) of photosystem II in *C. reinhardtii*.

1.2.1 Light-harvesting complexes at photosystem II initiate energy transfer

Photosystem II groups into megacomplexes consisting of multimers of PSII-LHCII supercomplexes (Iwai et al., 2008, Figure 1-3). A PSII supercomplex has a dimeric organization, with more than 60 proteins and around 300 chlorophylls serving two reaction centers (Croce and van Amerongen, 2014; Drop et al., 2014a). These pigments are organized densely and in a highly specific orientation into two moieties, the core complex where charge separation occurs and the outer antenna that enhances the absorption surface. The PSII core antenna consists of the chlorophyll *a* binding proteins CP43 and CP47, which are directly associated to the reaction center (Figure 1-3, dark green). The minor outer antenna, formed by chlorophyll *a* and *b* binding, monomeric LHCII CP29 and CP26, mediates exciton transfer to the core (Figure 1-3, magenta). CP24, found in higher plants, is missing in *C. reinhardtii* and it was only recently shown that its binding site is occupied by a trimeric LHCII termed N (naked, Drop et al., 2014a; Figure 1-3, red).

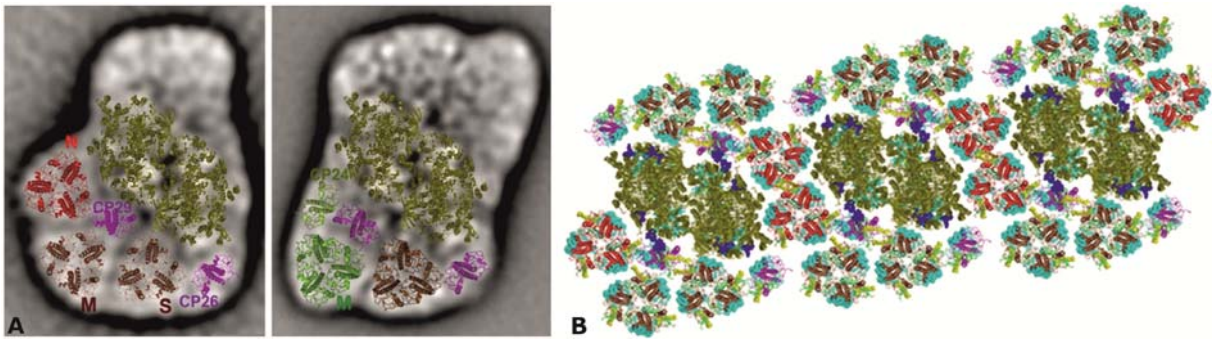

Figure 1-3 Organization of photosystem II in the green lineage.
(A) Comparison of a PSII supercomplex from *C. reinhardtii* (left) and *A. thaliana* (right). Assignment of the subunits in projection maps obtained from single particle electron microscopy showing PSII core (dark green), trimeric LHCII-S (brown) and LHCII-M (brown or light green, respectively), LHCII-N (red) as well as monomeric LHCII CP29, CP26 (magenta) and CP24 (light green). (B) Model of a PSII megacomplex consisting of three supercomplexes in $C_2S_2M_2N_2$ organization in *C. reinhardtii*. Color code as in A, left panel. Figures A and B were adapted from Drop et al. (2014a).

Intriguingly, while the PSII core is highly conserved from cyanobacteria to vascular plants (Nickelsen and Rengstl, 2013), the structure and regulation of the outer antenna significantly differ in between evolutionary lineages (Büchel, 2015; Ruban, 2015). The trimeric LHCII form the major outer antenna (Figure 1-3). Their excitation energy is assumed to be transported to the core via the minor antenna (Kouril et al., 2012; Dall'Osto et al., 2014), though direct transfer to the reaction center is generally possible (Sun et al., 2015). Similar to higher plants' supercomplexes, one LHCII per monomeric PSII is connected strongly (S) through interaction with the core, CP26 and CP29 and a moderately (M) bound trimer in *C. reinhardtii* (Figure 1-3, brown). The latter borders the S trimer and is relatively firmly associated to CP29 as well as trimer N in *C. reinhardtii* or CP24 in higher plants, respectively (Dainese and Bassi, 1991; Drop et al., 2014a). While core and minor antenna proteins are generally found in stoichiometric amounts to the reaction center, the amount of trimeric LHCII reaches up to three per monomeric core (1.2.2) (Tokutsu et al., 2012; Drop et al., 2014a). The PSII antenna of *C. reinhardtii* with a $C_2S_2M_2N_2$ supercomplex is hence notably larger than the largest one found in the model plant *Arabidopsis thaliana* with a $C_2S_2M_2$ organization (Caffarri et al., 2009).

LHCIIs are probably the most abundant membrane proteins on earth and account for approximately 70% of the pigments involved in photosynthesis (Croce et al., 1999). The nuclear encoded genes were among the first plant and algal genes to be cloned and sequenced (Bedbrook, 1980; Dunsmuir et al., 1982; Imbault et al., 1988). Biochemical analysis and crystallography of plant LHCII (Kühlbrandt et al., 1994; Liu et al., 2004; Standfuss et al., 2005) revealed that one monomer is organized into three transmembrane and two amphipathic α-helices, non-covalently binding 18 pigments: eight chlorophyll *a*, six chlorophyll *b*, two luteins or loroxanthins, one 9'-*cis*-neoxanthin and one xanthophyll cycle (1.2.2) substrate, violaxanthin or zeaxanthin (Grossman et al., 2004).

All LHCII belong to a superfamily of light harvesting proteins including also LHCI and LHC-like proteins. Because they share significant homology and common architecture, a monophylic origin is assumed (Wolfe et al., 1994). Nine trimer-forming LHCII isoforms (Teramoto et al., 2001; Elrad and Grossman, 2004), named LHCBM1 to LHCBM9 according to current nomenclature (Minagawa, 2009), group into four types which are unrelated to the three subfamilies defined in vascular plants (Elrad and Grossman, 2004). The roles of the different LHCBMs are not redundant, and distinct functions regarding the absorption of light, the distribution or dissipation of energy as well as membrane organization have been elucidated for certain isoforms (1.2.2).

1.2.2 LHCII proteins perform diverse roles in short-term photoprotection

Protective mechanisms on the level of light-harvesting decrease the excitation pressure on the photosystems by reducing the absorption cross section and dissipate excess energy (non-photochemical quenching, NPQ; Erickson et al., 2015; Figure 1-4). They can be classified by the time-scale of operation (Dietz, 2015), with (1) energy-dependent quenching (qE) switching LHCII from their light-harvesting into an energy dissipating mode within seconds; (2) the redistribution of LHCII between PSII and PSI termed state transition (qT) within minutes and (3) inhibition of photosynthesis (photoinhibition; qI) in the range of minutes to hours, occurring when the rate of PSII damage exceeds the rate of repair (Niyogi, 2009).

Energy-dependent quenching is considered a major photoprotective short-term response to high light stress (Iwai et al., 2007; Ruban et al., 2007), with 'high light' being undefined but generally referred to photon flux densities of 350 to 1200 μmol photons m^{-2} s^{-1} (Niyogi, 2009). When PET generated proton accumulation exceeds the rate of proton backflow into the stroma for ATP synthesis (1.1.1), the thylakoid lumen acidifies, which stimulates the induction of qE (Szabo et al., 2005; Niyogi and Truong, 2013). In consequence, LHCII dissociate from PSII and form aggregates in which excitation energy is safely dissipated as heat (Betterle et al., 2009; Figure 1-4). This quenching is intimately linked to energy transfer to xanthophylls, which reduces the half-life of excited chlorophylls. Therewith less chlorophyll molecules enter the triplet state through intersystem crossing, which prevents the subsequent formation of toxic singlet oxygen (Ruban et al., 2007). A *C. reinhardtii* mutant deficient in lutein and loroxanthin is impaired in the induction of qE by 50%, indicating a role of these xanthophylls in energy dependent quenching in this alga (Niyogi et al., 1997). The pH-gradient triggered deepoxidation of violaxanthin to zeaxanthin in the course of the xanthophyll cycle is of special importance for high energy quenching in higher plants (Yamamoto et al., 1962; Niyogi et al., 1997; Havaux and Niyogi, 1999), albeit it is not clear whether zeaxanthin contributes to qE through quenching activity or because its binding causes a conformational change of antenna complexes (Frank et al., 1994; Johnson et al., 2011).

A switch of antenna complexes from light-harvesting to energy-dissipating mode is likely triggered directly through protonation of luminal amino acid residues of LHCII or LHC-like proteins (Johnson et al., 2011; Tokutsu and Minagawa, 2013). In *C. reinhardtii*, LHCBM1 (Type IV) (Elrad et al., 2002; Ferrante et al., 2012) and the LHC-like protein LHCSR3 (Peers et al., 2009; Bonente et al., 2011; Tokutsu and Minagawa, 2013; Figure 1-4) were shown to be involved in energy-dependent quenching, and expression of LHCSR3 is only induced under stress conditions such as high light. LHCBM9, an antenna subunit regulated in a pattern contrasting the other LHCII, is involved in energy dissipation under nutrient deficiency stress conditions, though in a manner mechanistically differing from qE (Grewe et al., 2014). Interestingly, a gene product of PSBS has hitherto not been detected under various conditions in *C. reinhardtii* (Allmer et al., 2006; Bonente et al., 2008; Bonente et al., 2011). This non-pigmented four-helix protein of the LHC superfamily is crucial for qE in higher plants, and is, in contrast to LHCSR3, constitutively expressed (Niyogi and Truong, 2013). The different mechanisms of high energy quenching are thought to cause the generally large NPQ capacity vascular plants, while qE is intrinsically low and largely dependent on the growth condition in *C. reinhardtii* compared to plants (Finazzi et al., 2006; Bonente et al., 2012). State transitions often account for the major part of NPQ in this alga (Finazzi et al., 2006).

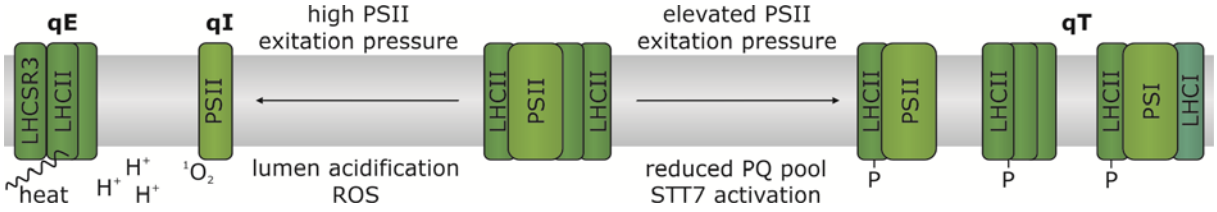

Figure 1-4 Short-term responses reducing PSII excitation pressure in *C. reinhardtii*.
Under highly excessive excitation, lumen acidification triggers energy dependent quenching (qE) to prevent oxidative damage and photoinhibition (qI). State transitions (qT) efficiently relieve PSII excitation pressure under moderate light conditions. A reduced PQ pool triggers STT7 activation which in turn causes the phosphorylation dependent detachment and migration of LHCII. The responses cannot be regarded as completely separate, but rather merging, with qE occurring within seconds and qT within minutes.

State transitions efficiently adjust the absorption cross section of photosystems I and II under low and moderate light intensities (Bonaventura and Myers, 1969; Murata, 1969; Rintamäki et al., 1997; Rintamäki et al., 2000; Figure 1-4), though a certain impact on high light acclimation was recently described (Allorent et al., 2013). Upon an imbalance favoring PSII excitation (state II conditions), an over-reduced PQ pool (Horton and Black, 1980), or more specifically the binding of plastoquinole to cyt b_6f (Zito et al., 1999), activates the state transition kinase STT7 which in turn leads to the phosphorylation of specific LHCII proteins (Bennett, 1977; Fleischmann et al., 1999; Depège et al., 2003; Lemeille et al., 2009; Lemeille et al., 2010; 1.3.1). In *C. reinhardtii*, up to 80% of the antenna subsequently dissociates from PSII and partially migrates to serve as an

efficient antenna for PSI (Delosme et al., 1996; Wientjes et al., 2013b; Nagy et al., 2014; Takahashi et al., 2014; Ünlü et al., 2014; see below). The extent of LHCII binding to PSI is currently under heavy debate, which is discussed in chapter 6 (6.3.1). However, state II transition of LHCII enhances cyclic electron flow around PSI (Finazzi et al., 2002; Iwai et al., 2010a; Lucker and Kramer, 2013), though both are stimulated independently (Takahashi et al., 2013). High light intensities both inactivate the state transition kinase through a thioredoxin mediated reduction of disulphide bonds (1.3.2) and lead to a decreased enzyme level (Lemeille et al., 2009; Puthiyaveetil, 2011; Wunder et al., 2012). Recently, also hydrogen peroxide was shown to hinder a state II transition (Roach et al., 2015), but the mechanism remains to be elucidated. The inactivation of the state transition kinase allows the reversion of qT through LHCII dephosphorylation by a permanently active phosphatase (Pribil et al., 2010; Shapiguzov et al., 2010; Rochaix, 2013). Overall, state transitions efficiently reduce PSII absorption cross section on the short-term, and are usually reversed within minutes to hours (Iwai et al., 2007; Lucker and Kramer, 2013).

In green algae and seed plants, different LHCII subunits were shown to have distinct and to some extend complementary roles during state transitions (Minagawa, 2011; Pietrzykowska et al., 2014). In *C. reinhardtii*, the first LHCII isoforms found associated to PSI under state II conditions were CP26, CP29, and LHCBM5 (type II) (Takahashi et al., 2006; Tokutsu et al., 2009). Later on, knock down of LHCBM2/7 (type III) was shown to reduce the ability to perform state transitions (Ferrante et al., 2012), implicating a role of these isoforms in state transitions. In a recent approach, applying very mild solubilization of thylakoids, all four types of LHCII were discovered at PSI (Drop et al., 2014b), interestingly with different phosphorylation patterns and kinetics (Iwai et al., 2008; Drop et al., 2014b). Phosphorylation of LHCII type I occurs early under state II conditions, and is thought to trigger the dissociation of the PSII megacomplex into supercomplexes (Iwai et al., 2008). Both phosphorylated LHCII type I and IV remain at PSII to some extent, similar to observations in *A. thaliana* (Wientjes et al., 2013a). LHCBM5 is the only isoform that has never been found associated to PSII, but which is part of very stable trimers (Drop et al., 2014a) forming an 'extra' LHCII pool (Wientjes et al., 2013a). Upon phosphorylation, trimers containing LHCBM5 bind to PSI, but interestingly the opposite is true for type III complexes (Drop et al., 2014b).

Despite the apparently strict classification of NPQ processes, high energy quenching and state transitions are not only complementary but overlap and show mechanistic similarities (Allorent et al., 2013; Erickson et al., 2015). Upon STT7 triggered phosphorylation, LHCSR3 co-migrates to PSI, presumably promoting energy dissipation during antenna movement (Niyogi and Truong, 2013). Under state II conditions, detached antenna complexes forms aggregates, similar to those typically found during qE (Ruban and Johnson, 2009; Tokutsu et al., 2009; Iwai et al., 2010b).

However, both processes relieve PSII excitation pressure on the short term. If excitation pressure remains high, long-term mechanisms involving expression control of light-harvesting proteins come into account.

1.2.3 LHCBM translation control in the context of long-term acclimation

The regulation of LHCBM protein levels determines light-harvesting capacity on the long-term. The absorption surfaces of both photosystems are influenced simultaneously because LHCII serves as an efficient antenna for PSII and PSI over a range of naturally occurring conditions (Wientjes et al., 2013b; Drop et al., 2014b). The outsourcing of genes encoding light-harvesting proteins from the chloroplast to the nuclear genome allows a complex multilevel control of LHCII expression (Woodson and Chory, 2008). After transcription in the nucleus and translation in the cytosol, the apoprotein is transported into the chloroplast where it is provided with pigments and folded into the thylakoid membrane (Figure 1-5B). Transport and retention of the apoprotein in the chloroplast as well as folding of the complex impact LHCII abundance and are described elsewhere (Park and Hoober, 1997; Bellafiore et al., 2002; Stengel et al., 2009; Kirst et al., 2012; Mitra et al., 2012). Here, emphasis is laid on expression regulation outside the chloroplast, namely control of transcript abundance and protein synthesis.

LHCII transcription control has been studied in various photosynthetic organisms over decades, and many factors are now known to influence transcript abundance, including circadian rhythm (Paulsen and Bogorad, 1988; Jacobshagen et al., 1996), acetate supply (Kindle, 1987), and light intensity (Johanningmeier, 1988; Escoubas et al., 1995; Teramoto et al., 2002; Elrad and Grossman, 2004; Humby et al., 2009). Despite extensive research, the signaling molecules affecting transcription are still under debate (1.3). For some species, a rather clear picture could be obtained. For example in the green alga *Dunaliella*, the trans-thylakoid membrane potential influences LHCII transcription on short time scales, whereas on time scales of more than eight hours, regulation involves a kinase cascade coupled to the PQ pool redox state (Escoubas et al., 1995; Maxwell et al., 1995; Chen et al., 2004).

In contrast, a complex and not fully understood transcriptional and post-transcriptional regulation on the expression of light-harvesting proteins is observed in *C. reinhardtii*. Under excess light, transcription of LHCII genes is down-regulated within two hours, but mRNA abundance reaches low light levels again after six to eight hours (Durnford et al., 2003; Elrad and Grossman, 2004). Interestingly, the repression of LHCBM transcription under elevated light occurs even when electron transfer to plastoquinone is blocked and in the absence of both PSII and PSI, indicating that the PQ redox state is not the dominant cue regulating LHCII mRNA levels in this alga (Teramoto et al., 2002; Humby et al., 2009). PQ independent signaling might instead come

into account. Light sensing via photoreceptors was shown to be involved in regulating the mRNA level of LHC-like proteins (Gagné and Guertin, 1992; Teramoto et al., 2006), and transcription of LHCBM6 is controlled by the blue-light receptor phototropin during a transition from darkness to very low light (Im et al., 2006). Also chlorophyll precursors such as magnesium protoporphyrin IX are involved in light dependent retrograde signaling (Formighieri et al., 2012; Brzezowski et al., 2014) and were shown to influence LHCBM mRNA levels in *C. reinhardtii* (Johanningmeier and Howell, 1984; Johanningmeier, 1988; 1.3).

Because translation control permits a faster response than transcription regulation (Dietz, 2015), the rather rapid changes in redox poise might be sensed and implemented on the level of protein biosynthesis. Indeed, there is growing evidence for redox mediated post-transcriptional control in higher plants as well as green algae (Frigerio et al., 2007; Petracek et al., 1997; McKim and Durnford, 2006; Wobbe et al., 2008; Wobbe et al., 2009). Organellar gene expression is mainly regulated post-transcriptionally and involves nuclear encoded proteins (Wobbe et al., 2008; Woodson and Chory, 2008). However, factors regulating translation of light-harvesting proteins in the cytosol are unknown for most species.

The identification of the cytosolic nucleic acid binding protein 1 (NAB1) as a repressor of LHCBM protein biosynthesis in *C. reinhardtii* (Mussgnug et al., 2005) provided new insights into translation control of nuclear encoded photosynthesis associated genes. NAB1 stabilizes transcripts of LHCII at the preinitiation level, with a strong preference for *LHCBM6*, therewith sequestering the mRNA and repressing translation (Figure 1-5). The knock out of this repressor leads to a dark green, large antenna phenotype of the mutant, which is characterized by an increased accumulation of light-harvesting proteins and chlorophyll content (Figure 1-5A), although at the same time *LHCBM6* mRNA levels are reduced to 30% compared to the wild-type (Mussgnug et al., 2005). Particularly after an increase in light intensity from 40 to 200 µmol photons m^{-2} s^{-1}, NAB1 mediated translation control accounts for half of the observed decrease in LHCBM6 protein level. Growth of the mutant is in consequence impaired under moderate high light, and a decreased PSII quantum yield in the light indicates inefficient energy transduction under these conditions (Mussgnug et al., 2005).

The NAB1 protein consists of an N-terminal cold shock domain (CSD) and a C-terminal RNA recognition motif (RRM) domain, connected by a loop containing glycine arginine rich motifs (GAR motif I and II) (Figure 1-5C).

Though the combination of two RNA binding domains in one protein is found regularly, the combination of CSD and RRM is unique; and NAB1 is furthermore the only protein with a CSD in *C. reinhardtii* (Graumann and Marahiel, 1998; Mussgnug et al., 2005). NAB1 type proteins are hitherto only found in close relatives to *C. reinhardtii*: *C. incerta* and *Volvox carteri* (Nematollahi et al., 2006; Popescu et al., 2006). While the CSD is crucial for the specific binding of NAB1 to *LHCBM6* mRNA (Mussgnug et al., 2005), post-translational modifications within the other domains regulate the activity of the protein, including methylation of two arginines in GAR motif I with relatively slow dynamics and fast, redox based cysteine modifications in the RRM domain (Wobbe et al., 2009; Blifernez et al., 2011).

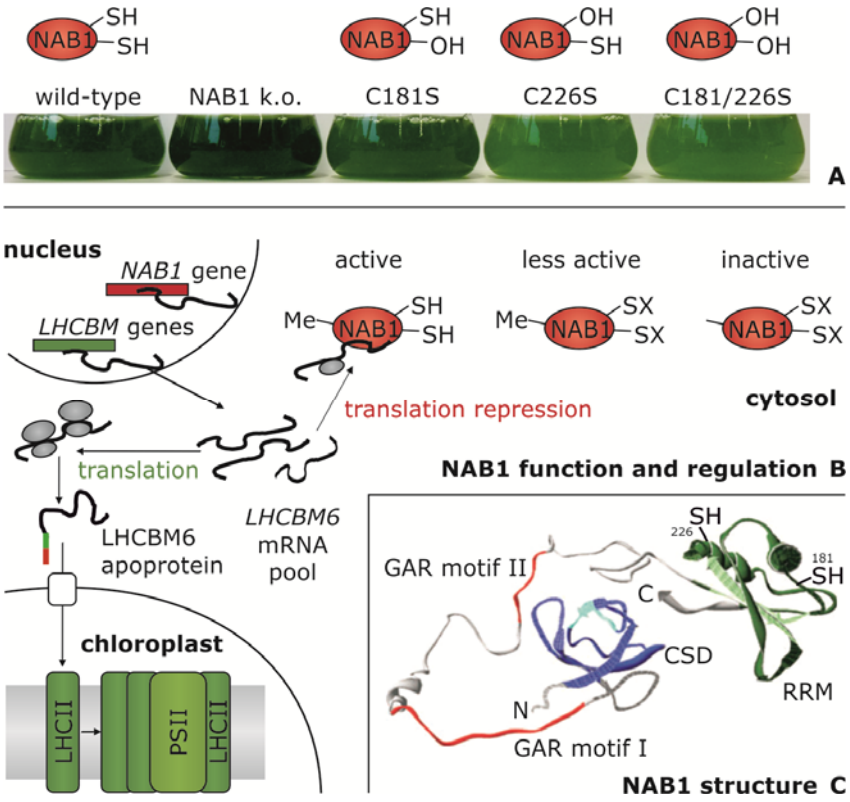

Figure 1-5 LHCBM translation repression mediated by the cytosolic RNA binding protein NAB1.
(A) Appearance of *C. reinhardtii* cultures carrying no (NAB1 k.o.) or modified (C181S and/or C226S) versions of NAB1, compared to wild-type cells. (B) Function of NAB1 and post-translational activity regulation by arginine methylation (Me) and cysteine modification (SX). (C) Model of NAB1 structure (Blifernez et al., 2011) with an N-terminal cold shock domain (CSD, blue), two glycine arginine rich (GAR) mofifs (red) and a C-terminal RNA recognition motif (RRM) domain (green), which harbors cysteines (SH) at position 181 and 226.

The RRM domain is crucial for a fine-tuning of repressor activity (Wobbe et al., 2009). The thiol state of the two cysteines at amino acid position 181 and 226 confers high RNA binding activity of NAB1, in consequence increasing translation repression, and cysteine oxidation decreases binding affinity (Figure 1-5B). Replacement of the cysteines by serines mimics a reduced, permanently active state and leads to a pale green, small antenna phenotype of mutant cultures, with the C226S single mutation and double mutation causing a stronger phenotype than C181S single mutation (Figure 1-5A). Under high light conditions, the smaller antenna is more efficient,

causing an increased light to biomass conversion of a mutant that contains a constitutively expressed C181/226S version of NAB1 in addition to the native protein (Beckmann et al., 2009).

However, the mechanism of cysteine oxidation and reduction remained to be elucidated. As the formation of intramolecular disulphide bridges was ruled out (Wobbe et al., 2009), the reversible addition of S-reactive groups such as glutathione or nitric oxide and redox-protein mediated re-reduction should be considered (1.3.1; 1.3.2). The identification of modifying agents and enzymes would provide new links to signaling pathways that control light-harvesting protein synthesis.

1.3 Interorganellar communication

Compartmentalization of eukaryotic cells requires intensive interorganellar communication, and gene expression in the nucleus, chloroplast and mitochondria occurs highly coordinated to enable cellular hemostasis (Woodson and Chory, 2008; Grimm et al., 2014). By definition, the nucleus integrates retrograde signals emerging from the other organelles, and expression of nuclear encoded photosynthesis associated genes is heavily influenced by cues from the chloroplast (Dietz, 2015). Light-harvesting proteins are encoded in the nucleus, synthesized in the cytosol and function in the chloroplast thylakoid membrane, allowing and demanding a complex multilevel regulation to ensure optimal light energy input. Extensive research on signals controlling nuclear photosynthetic genes has identified numerous candidate molecules and pathways, but unambiguous evidence is still missing (Leister, 2012). Furthermore challenging, though retrograde signals are used by all eukaryotes, there is only little interspecific conservation, indicating that the pathways continuously evolve to suit the demands of the organisms (Woodson and Chory, 2008).

Signaling pathways are highly complex and may be classified according to the severity of stress and metabolic imbalance under which they emerge (Dietz, 2015). Stress, such as abrupt and intense variations in light intensity or temperature, drought, and pathogens, often creates an oxidative burden. Under these conditions, reactive oxygen species (ROS) such as singlet oxygen and hydrogen peroxide (Figure 1-6; $^1O_2$ and H_2O_2) as well as metabolites of the tetrapyrrole pathway are involved in the induction of antioxidant defense and repair systems (Suzuki et al., 2012; Fischer et al., 2013; Brzezowski et al., 2014), but also programmed cell death may be induced (Murik et al., 2014; Sirisha et al., 2014). Comprehensive descriptions on retrograde signaling under stress can be found in numerous current reviews (e.g. Foyer and Noctor, 2009; Kleine et al., 2009; Pfannschmidt, 2010; Rochaix, 2013; Dietz, 2015). In this work, less stressful conditions were studied, dealing with environmental changes that induce photosynthetic and metabolic adjustment rather than severe damage. Signals which come into question include the sensing of cellular redox poise and metabolite levels.

1.3.1 Retrograde signals emerging from the chloroplast

It is the 'grand design' of photosynthesis that photosynthetic performance itself serves as signal generator for adjustments of cellular metabolism and gene expression (Anderson et al., 1995; Brautigam et al., 2009). Several concepts were postulated on the nature and transduction of primary cues, from signals diffusing passively towards the site of action to signaling cascades and controlled shuttling, which allow a more complex regulation of gene expression (Leister, 2012). Because photosynthesis heavily impacts the cellular energetics, commonly proposed cues are redox signals, including the reduction state of electron transport chain and cues downstream of PSI, as well as the abundance of metabolites and end products; all thought to act in concert (Koussevitzky et al., 2007; Brautigam et al., 2010; Dietz, 2015).

The concept of cellular redox poise mainly applies to soluble redox couples with a relatively slow turnover of seconds to minutes. The redox state of NADPH/$NADP^+$ is directly affected by photosynthetic electron transport (Figure 1-6; 1.1.1), while other reductants such as glutathione and ascorbate are generally kept in a highly reduced state (Foyer and Noctor, 2009). Reducing power is indirectly exported from the chloroplast via transport systems like the malate oxaloacetate shuttle (Figure 1-2, MAL, OAA). If NADPH converting enzymes and transporters are regulated, molecules affecting their activity might as well be considered as 'true' primary signals (Leister, 2012). Indeed, increasing $NADP^+$ concentrations inhibit oxaloacetate reducing activity of malate dehydrogenase, and reduced thioredoxins counteract this inhibition, allowing a feedback and feed-forward regulation (Scheibe and Jacquot, 1983). Exported reducing power may be exploited in other compartments directly as substrate for anabolic reactions or for the reduction of redox proteins (Figure 1-6; 1.1.1; 1.3.2), consequently altering metabolism and influencing nuclear gene expression (Scheibe and Dietz, 2012).

Components in the photosynthetic electron transport chain turn over on a sub-second scale with a rapidly changing redox state (Foyer and Noctor, 2009). The redox state of the plastoquinone pool is in the focus of numerous studies, as it sensitively reacts to environmental changes such as light (Fey et al., 2005) or carbon dioxide and oxygen availability (Dietz et al., 1985). It is generally accepted that the redox state of the PQ pool influences plastid gene expression (Danon and Mayfield, 1994; Pfannschmidt, 2003), while its role in the regulation of nuclear transcription is still inconclusive (Humby et al., 2009). LHCII state transitions are clearly linked to the PQ redox state (Figure 1-6; 1.2.2), and in *A. thaliana*, the state transition kinase was shown to impact long-term acclimation processes such as the ratio of photosystem I and II (Bonardi et al., 2005; Pesaresi et al., 2009), especially in developing seeds (Tikkanen and Aro, 2014). In *C. reinhardtii*, involvement of the state transition kinase STT7 in long-term photosynthetic regulation is less

clear, as the knock out mutant did not exhibit any impairment under various growth conditions unless respiration was artificially diminished (Cardol et al., 2009). More recently, the mutant was shown to react sensitively to a sudden rise in incident light intensity (Allorent et al., 2013). However, it remains unclear whether this phenotype is due to physiological alterations or impaired signaling, or both.

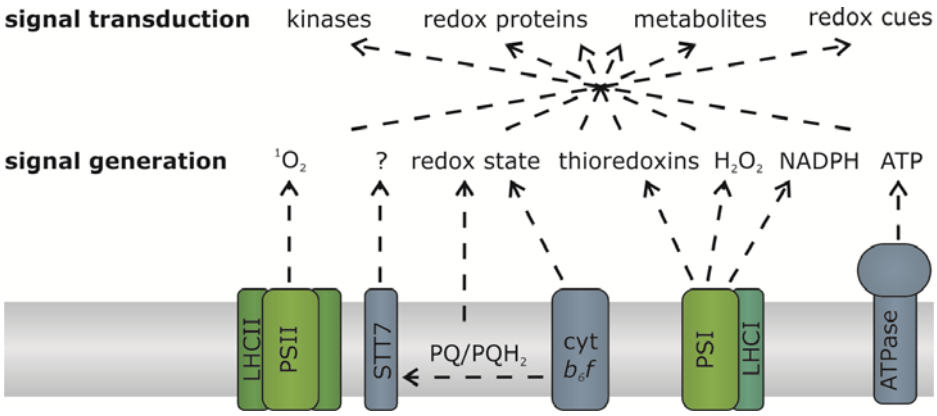

Figure 1-6 Photosynthetic signal generation and transduction.
Candidate cues emerging from photosynthesis and transduction pathways to other cellular compartments are shown. Some signals may converge during transmission allowing coordinated sensing of cellular states.

Reactive oxygen or nitrogen intermediates emerge naturally in consequence of aerobic metabolism in several cellular compartments, but severe stress implicates an oxidative burst that might cause severe cell damage (Del Río et al., 2003; Foyer and Noctor, 2009). Some reactive species such as nitric oxide (NO) were shown to be involved in signaling beyond high light stress conditions or plant immune responses (Wei et al., 2014). NO based signaling is intensively studied in mammals, which generate nitric oxide via NO synthase (NOS) through the oxidation of arginine to citrulline, and impaired nitrosylation is involved in the pathology of numerous diseases (Martínez-Ruiz et al., 2012; Majmudar and Martin, 2014). In plants, nitric oxide generation was discovered early on (Klepper, 1979), but its source remains to be unambiguously identified (Moreau et al., 2010). Proposed enzymatic systems include nitrate reductase, generating NO as a side reaction, and arginine dependent synthases similar to animal NOS (Corpas et al., 2009; Xie et al., 2013; Wei et al., 2014), and chloroplasts, mitochondria and peroxisomes are thought to evolve NO (Del Río et al., 2003; Raghavendra and Padmasree, 2003). Regardless of the synthesizing pathway, it is now clear that nitric oxide is involved in the regulation of numerous genes in plants and algae, including photosynthesis associated genes (Morisse et al., 2014; Wei et al., 2014), and signaling is mediated via direct interaction with protein metal centers or reversible nitrosylation of cysteine and tyrosine residues (Moreau et al., 2010; 1.3.2).

Redox and metabolic control are highly intertwined and mutually dependent (Brautigam et al., 2009; Geigenberger and Fernie, 2014). Changes in metabolism provide detailed information

about the state of the cell over a range of time periods (Dietz, 2015) and interorganellar exchange of metabolites allows the integration of photosynthetic performance with for instance energy and carbon conversion in the mitochondria (Raghavendra and Padmasree, 2003; Humby et al., 2009; Schwarzländer and Finkemeier, 2013; 1.1; Figure 1-2). Indeed it was shown that carbon dioxide availability influences expression of photosystem I and II reaction center subunits in , with metabolic control being dominant to the sensing of the redox state of the PQ pool (Wormuth et al., 2006). Commonly proposed signaling cues include metabolites of carbohydrate, lipid and protein ana- and catabolism as well as intermediates of pigment metabolisms such as the chlorophyll precursor magnesium protoporphyrin IX or oxidation products of β-carotene such as β-cyclocitral (Leister, 2012; Dietz, 2015).

1.3.2 Transduction and implementation of retrograde signals

Redox cues and metabolites are exported from the chloroplast via transport systems like the triose phosphate and the malate oxaloacetate shuttle (Scheibe and Dietz, 2012; 1.1.1; Figure 1-2) with the key enzymes NADPH dependent malate dehydrogenase and GAP dehydrogenase (Hoefnagel et al., 1998; Foyer and Noctor, 2009). Such shuttles fulfill a double function, as they both relieve electron pressure in the chloroplast and signal the reduction state to the cytosol and nucleus (Foyer and Noctor, 2009). Here the signals may implicate post-translational modifications of proteins, and predominantly studied in regulation of photosynthesis are phosphorylation and cysteine modification, as well as their interplay (reviewed in Rochaix, 2013).

Phosphorylation influences protein structure, function, and localization, and is therefore involved in adjustment of manifold cellular processes (Slade et al., 2014). The phosphoproteom of chloroplasts is complex and intensively studied, with the phosphorylation of photosystems under high excitation pressure (Bonardi et al., 2005) and of LHCII triggering state transitions (1.2.2) as prominent examples. In *C. reinhardtii*, kinase cascades putatively transducing signals in the other organelles are poorly understood. Fast retrograde signaling through triose phosphate shuttle and mitogen-activated protein kinases is common in higher plants (Vogel et al., 2014). *C. reinhardtii* indeed encodes six genes for mitogen-activated protein kinases (Mohanta et al., 2015), and there is evidence for their involvement in signaling controlling lipid and carotenoid metabolism (Choi et al., 2015).

The implementation of reducing or oxidizing cues has been extensively studied. Rather mild oxidative and nitrosative species such as hydrogen peroxide and nitric oxide cause the generally reversible oxidation of specific cysteine residues and are therefore thought to be important for redox based signaling pathways (Spadaro et al., 2010; 1.3.1), while irreversible cysteine oxidation

occurs under oxidative stress (Akter et al., 2015). Major reversible cysteine modifications include thiolation and nitrosylation. Both were recently studied intensively in *C. reinhardtii*, and many proteins performing or regulating photosynthesis were found to be regulated in this manner (Michelet et al., 2008; Zaffagnini et al., 2012; Morisse et al., 2014).

Thiolation describes the formation of disulfide bridges within and in between proteins, or addition of smaller thiols such as the tripeptide glutathione. S-Glutathionylation was long thought to occur as a side reaction under oxidative and nitrosative stress (Grek et al., 2013). However, the specific S-glutathionylation of a chloroplastic thioredoxin (Michelet et al., 2005) and GAP dehydrogenase (Zaffagnini et al., 2007) strengthens its role as an important redox modification occurring under stress conditions (Zaffagnini et al., 2012).

Nitrosylation is well characterized in animals compared to photosynthetic organisms, but recently increasing interest fuelled fast growing knowledge about this modification in plant systems (Lamotte et al., 2015). The source of nitric oxide and enzymes catalyzing nitrosylation are hitherto not clearly identified in plants and algae (Spadaro et al., 2010; 1.3.1). NO can react with glutathione to S-nitrosoglutathione (GSNO), which might in turn be involved in trans-nitrosylation reactions. The level of GSNO is controlled by S-nitrosoglutathione reductase (Liu et al., 2001). As a relatively stable molecule, S-nitrosoglutathione might also function in storage of nitric oxide (Feechan et al., 2005). Despite incomplete knowledge on how S-nitrosylation occurs, this modification is regarded as a major regulatory mechanism, and a proteomic approach recently identified 492 nitrosylated proteins in *C. reinhardtii* (Morisse et al., 2014).

The removal of cysteine modifications is a key component of signal transduction as well. While glutaredoxins reduce mixed disulphides and therefore catalyze deglutathionylation reactions amongst others (König et al., 2012), thioredoxins (TRX) were shown to denitrosylate cysteines in higher plants and mammals (Benhar et al., 2008; Kneeshaw et al., 2014). Glutaredoxins and thioredoxins belong to the TRX superfamily of ubiquitous antioxidant enzymes which are well known to reduce modified cysteine residues (Lemaire and Miginiac-Maslow, 2004; König et al., 2012). They are involved in the regulation of central enzymes of carbon metabolism (Serrato et al., 2013; Daloso et al., 2015) as well as light-harvesting via de-activation of LHCII state transitions (Puthiyaveetil, 2011; 1.2.2), but also translation factors are targets for cytosolic redox proteins in plants (Yamazaki et al., 2004; Rouhier et al., 2005).

C. reinhardtii encodes eight thioredoxins with specific subcellular localizations (Lemaire and Miginiac-Maslow, 2004). Chloroplastic thioredoxins are reduced in the light by ferredoxin dependent thioredoxin reductase, while the cytosolic isoforms, named TRX h, are reduced by NADPH dependent thioredoxin reductase (NTR). Two cytosolic thioredoxins are found in

C. reinhardtii with specialized, non-redundant functions (Sarkar et al., 2005), and TRX h1 is the generally more abundant form (Lemaire and Miginiac-Maslow, 2004).

The modification of proteins influences cellular metabolism, as exemplarily described above, and alters gene expression. Intriguingly, an increasing number of metabolic enzymes are shown to bind nucleic acids and therewith directly impact transcription or translation (Hara et al., 2005; Cieśla, 2006; Bohne et al., 2013), further strengthen the close linkage of metabolism and gene expression.

The nucleus integrates signals of several organelles (Pfannschmidt, 2010). While transcript abundance of some genes changes very fast and such early responses use preexisting signaling pathways and transcription factors, transcript levels of other genes change with a delay (Dietz, 2015). Retrograde signals influence nuclear gene expression through interaction with transcription factors in the nucleus or with proteins that migrate into the nucleus. However, information on regulatory elements and factors controlling transcription in *C. reinhardtii* is rare, and analysis of nuclear promotors helps elucidating signal perception and implementation mechanisms.

1.3.3 Promoter studies allow analysis of chloroplast to nucleus signaling

The understanding of promoter regions in the nuclear genome of *C. reinhardtii* is still in its infancy. An *in silico* analysis comparing the local distribution of short sequences (Yamamoto et al., 2007) revealed that the general structures apparently differ from those found in mammals or the model plant *A. thaliana* (Wimalanathan, 2011). While in the genes of these organisms the TATA-box or an initiator region is frequently found as crucial promoter element, the transcription start site is proposed to be a region more generally adenine thymine rich rather than a specific sequence motif for many *C. reinhardtii* genes (Kadonaga, 2012; Wimalanathan, 2011; Yang et al., 2007; Yamamoto et al., 2007).

However, promoters with a comparatively clear expression pattern have been experimentally investigated in this alga, and for some genes TATA-boxes or initiator like sequences as well as *cis*-regulatory elements (CREs) could be identified. The gene of the chaperone HSP70A was analyzed in depth, as the promoter facilitates heterologous protein expression in *C. reinhardtii* (Müller et al., 1992; Lodha et al., 2008), and the factors that counteract transgene silencing processes of the cell were recently identified (Strenkert et al., 2013). Interestingly, the transcription induction of *HSP70A* through light is mimicked by magnesium protoporphyrin IX, and a corresponding plastid response element was found between two promoters, one of which containing a classical TATA-box (von Gromoff et al., 2006).

Another well-studied promoter belongs to the *CAH1* gene encoding a periplasmic carbonic anhydrase. Transcription is induced under limiting CO_2 conditions as part of the carbon concentrating mechanism (Spalding, 2009; Wang et al., 2011; 1.1.2). Promoter analysis revealed enhancing and silencing CREs, and proteins interacting with two enhancer elements could be detected via gel mobility shift assays (Kucho et al., 1999; Kucho et al., 2003). As another example, the glutathione peroxidase homologous gene is specifically upregulated by singlet oxygen generated by PSII during high light and CREs that confer the responsiveness to singlet oxygen could be identified (Leisinger et al., 2001; Fischer et al., 2005; Fischer et al., 2006). Notably, transcription starts at two alternative sites, either a TATA-box or an initiator core promoter, which results in dual targeting of the peroxidase to the chloroplast or cytosol (Fischer et al., 2009). Also elements responsible for light and ammonium dependence of the transcription of the nitrate reductase encoding gene were approached (Loppes and Radoux, 2001).

For genes encoding light-harvesting proteins, the results are less clear. Hahn and Kück (1999) analyzed the *LHCBM6* promoter and found that a 122 bp element upstream of the translation start is sufficient to drive transcription and only weakly responses to changes in light regime. With the addidtional -122 to -255 bp region, the transcriptional response to light is restored, suggesting that this part contains crucial regulatory elements. Comparison with sequences of other light-dependent promoters suggested several CREs, but the conclusions were questioned later on, as these elements can also be found in genes that do not respond to light (Elrad and Grossman, 2004). Later on, the regulation of the *LHCBM6* promoter was analyzed thoroughly and the study revealed that metabolic changes rather than the PQ reduction state signal the demand for light-harvesting proteins to the nucleus (Humby et al., 2009). LHCBM9 is regulated in a manner contrasting the other LHCII subunits. Under nutrient depletion, the expression of this isoform increased (Grewe et al., 2014), and very recently sulfur responsive elements on the *LHCBM9* promoter were approached through *in silico* and reporter studies (Sawyer et al., 2015; see below).

Information about the promoter and expression regulation of the LHCII translation repressor NAB1 is rare. Apparently, a 800 bp sequence upstream the translation start contains essential elements, as this fragment was sufficient to drive complementation of the NAB1 knock out mutant (Mussgnug et al., 2005). As nuclear gene expression is usually regulated on the transcriptional level (Harris, 2009), *NAB1* promoter activity is likely controlled as well. Transcriptome studies indeed revealed changes in transcript abundance dependent on carbon dioxide supply (Winck et al., 2013a), while a mutant with impaired tetrapyrrole signaling exhibited only slightly altered *NAB1* mRNA levels (Formighieri et al., 2012). Such analyses reinforce the

assumption that the repressor protein is, in addition to post-translational control (1.2.3), regulated on the level of transcription, and the investigation of NAB1 expression is part of this thesis.

Several *in silico* tools are available to analyze promoters and predict *cis*-regulatory elements (CRE) in plants, PlantCARE (Lescot et al., 2002) and PLACE (Higo et al., 1999), as well as MERCED for octamer sequences (Ding et al., 2012). These tools indicate potential regulatory sites and therefore help narrowing down sites of interest, as for instance exploited in the study of the *LHCBM9* promoter (Sawyer et al., 2015).

Empirical data however provides more direct insights, and many of the results mentioned in this section were obtained by exploiting reporter constructs which mediate the expression of an easily detected and quantified protein driven by the promoter of interest (Hallmann, 2007). Such systems have generally been widely used for studying gene expression, as they allow a rapid and high-throughput screening of transformants and an efficient analysis of virtually any promoter (Roda et al., 2004; Gahan, 2012; Rosellini, 2012).

Several reporter systems were employed and refined for use in *C. reinhardtii*. The native arylsulfatase was used early on (Davies et al., 1992; Hahn and Kück, 1999), as the enzyme catalyzes a quantifiable color-forming reaction and is naturally not expressed unless sulfur is limiting. This system has some disadvantages though, including slowness of response, comparatively low sensitivity and high stability of the protein (Fuhrmann et al., 2004). To counter this, the luciferase gene from the soft coral *Renilla reniformis* was used (Fuhrmann et al., 2004; Heitzer and Zschoernig, 2007), using bioluminescence as signal. The construct was subsequently improved applying the luciferase gene of the marine copepod *Gaussia princeps* optimized for use in *C. reinhardtii* (Ruecker et al., 2008; Shao and Bock, 2008). The relatively small, 185 amino acids containing *Gaussia* luciferase (*g*Luc) converts the luciferin coelenterazine in a light emitting reaction with an emission maximum at 470 nm. Most important for the use as reporter, the emerging signal is extraordinarily bright and linear to protein concentration over five orders of magnitude (Verhaegen and Christopoulos, 2002).

Expression of *Gaussia* luciferase from the nucleus allows the export of the reporter protein into the culture supernatant, as the gene contains a secretion signal recognized by *C. reinhardtii* cells (Ruecker et al., 2008). This greatly facilitates luminescence quantification as shading by algal pigments as well as background chlorophyll fluorescence is circumvented. As the signal peptide was not optimized for secretion by algal cells, a new reporter construct combining the *g*Luc system with a *C. reinhardtii* secretion signal could enhance protein export and therewith facilitate expression analysis of photosynthesis related proteins encoded in the nucleus.

2 Objective

Balanced light capture ensures optimal photosynthetic performance, and expression control on nuclear encoded light-harvesting proteins (LHCII) is therefore a key element of acclimation responses to fluctuating environments (1.2). In this thesis, the adjustment of light-harvesting at photosystem II (PSII) and underlying intracellular communication in the model green alga *Chlamydomonas reinhardtii* was analyzed using the LHCII translation repressor NAB1 as a tool. This cytosolic protein sequesters transcripts of nuclear encoded LHCII, thus controlling light-harvesting protein synthesis (Mussgnug et al., 2005). Detailed studies led to a working model of post-translational NAB1 activity control by both reversible redox based cysteine modification (Wobbe et al., 2009) and arginine methylation (Blifernez et al., 2011). However, the conditions triggering LHCBM translation repression, the mechanism of redox activity control, and the involvement of NAB1 expression control had yet to be elucidated. To understand the regulatory network adjusting light-harvesting at PSII, uncovering the interorganellar signalling pathways which concert short- and long-term acclimation responses is of significant importance.

In particular, the work presented here addresses the following issues:

- Under which environmental and physiological conditions does NAB1 mediated translation control adjust light-harvesting antenna sizes?
- Does NAB1 expression regulation influence LHCII translation control in addition to post-translational activity switches?
- Which molecular mechanisms and signaling pathways underlie the redox based NAB1 activity control?
- How do short- and long-term regulatory responses interrelate to adjust PSII light capture?
- How do chloroplast, cytosol, and nucleus communicate to coordinate light-harvesting regulation?

In order to answer these questions, methods from molecular biology, biochemistry, biophysics and physiology were combined. The regulation of nuclear transcription of photosynthesis related genes was examined by analyzing the *NAB1* promoter *in silico* and *in situ*. A reporter system developed in this work was applied to this end, and *cis*-regulatory regions were systematically narrowed down. Long-term control of light capture was investigated taking transcript and protein levels as well as growth performances of mutant *C. reinhardtii* cell lines into account. Chlorophyll fluorescence analyses revealed information about short-term responses, PSII light-harvesting antenna size and photosynthetic electron flow. Eventually, the novel findings were integrated into a model depicting distinct PSII excitation pressure states in response to light and carbon availability and a sophisticated multi-level regulation which adjusts photosynthetic light capture.

3 A versatile reporter system to analyze nuclear promoters in *C. reinhardtii*

3.1 Significance

The majority of photosynthesis associated proteins are encoded in the nucleus (1.3). Organellar and nuclear gene expression needs to be coordinated, and there is ongoing research on how retrograde signals emerging from the chloroplast control promoter activity in the nucleus (1.3.3). In order to facilitate the analysis of nuclear promoters in *C. reinhardtii*, a vector system was developed with a codon optimized *Gaussia* luciferase gene driven by the promoter of interest, which enables efficient secretion of the reporter protein. This allowed rapid screening of transformant cell lines and promoters analysis. The results were obtained in collaboration as outlined below, and published in Lauersen et al. (2013).

3.2 Publication I

Efficient recombinant protein production and secretion from nuclear transgenes in *Chlamydomonas reinhardtii*

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published in

Journal of Biotechnology 167:101-110 (2013)

Author contributions

The experiments were designed by Kyle J. Lauersen, Hanna Berger, Jan H. Mussgnug, and Olaf Kruse.

The experiments were performed by Kyle J. Lauersen and Hanna Berger. Kyle J. Lauersen coordinated the work and was responsible for the genetic engineering of the *C. reinhardtii* strains, screening, and identification of the mutants presented in this work (Figures 3-1 and 3-2). Kyle J. Lauersen also performed the adaptation of bioluminescence (Figure 3-3) and dot-blot analyses (Figure 3-4B). Hanna Berger performed the demonstration of recombinant protein accumulation by gel electrophoresis and immunoblot analysis (Figures 3-4A and 3-5), and was responsible for nickel affinity chromatography (Figure 3-6).

Data was analyzed and interpreted by Kyle J. Lauersen, Hanna Berger, Jan H. Mussgnug, and Olaf Kruse. The manuscript was written by Kyle J. Lauersen, Hanna Berger, Jan H. Mussgnug, and Olaf Kruse.

Abstract

Microalgae are diverse photosynthetic microbes which offer the potential for production of a number of high value products (HVP) such as pigments, oils, and bio-active compounds. Fast growth rates, ease of photo-autotrophic cultivation, unique metabolic properties and continuing progress in algal transgenics have raised interest in the use of microalgae systems for recombinant protein (RP) production. This work demonstrates the development of an advanced RP production and secretion system for the green unicellular model alga *Chlamydomonas reinhardtii*. We generated a versatile expression vector that employs the secretion signal of the native extracellular *C. reinhardtii* carbonic anhydrase for efficient RP secretion into the culture medium. Unique restriction sites were placed between the regulatory elements to allow fast and easy sub-cloning of sequences of interest. Positive transformants can rapidly be identified by high-throughput plate-level screens via a coupled *Gaussia* luciferase marker. The vector was tested in *Chlamydomonas* wild type CC1883 (wt) and in the transgene expression transformant UVM4. Compared to the native secretion signal of the *Gaussia* luciferase, up to 84% higher RP production could be achieved. With this new expression system we could generate transformants that express up to 10 mg RP per liter culture without further optimization. The target RP is found exclusively in culture medium and can therefore easily be isolated and purified. We conclude that this new expression system will be a valuable tool for many heterologous protein expression applications from *C. reinhardtii* in the future.

Introduction

The study of genetic modification has come far from the first understanding of chemical and radiation induced mutagenesis (Auerbach and Robson, 1944; Demerec and Latarjet, 1946; Witkin, 1947) and the first successful transformation of a foreign vector into a bacterial host (Cohen et al., 1973) to the current wealth of knowledge available to researchers. Since the advent of foreign protein production in *Escherichia coli* (Itakura et al., 1977) the idea-scape surrounding RP expression has exploded into a vibrant mosaic of host organisms, targets, and strategies. Today, recombinant expression systems exist for species from most kingdoms while transformation strategies and host ranges are accelerating rapidly with the advent of flexible synthetic DNA technologies. Chimeric gene expression techniques are valuable for functional analysis of cellular mechanics, modifying traits of host organisms, and the production of high value bio-products. Genetic manipulation is possible in many bacterial (Terpe, 2006; Sahdev et al., 2008), mammalian (Chu and Robinson, 2001; Schmidt, 2004), plant (Fischer et al., 2004; Manuell et al., 2007), insect (Ikonomou et al., 2003), yeast (Porro et al., 2005), and fungal (Talabardon and Yang, 2005) systems.

Bacterial hosts are widely used for RP production (Terpe, 2006), however, lack eukaryotic post translational modification capabilities and may require complicated processing steps to handle purification of proteins from inclusion bodies (Sahdev et al., 2008). Yeast based systems present an interesting alternative to prokaryotic expression systems, demonstrating basic eukaryotic machinery, known genetics, ease of handling, and the capacity for protein secretion (Porro et al., 2005). However, yeasts maintain species specific post-translational modification and secretion mechanisms often result in insufficient protein yields (Porro et al., 2005). Insect cell expression systems also maintain variable post-translational modification properties and can be limited by costs and technical difficulties of scale-up as well as the inherent lytic nature the baculovirus-based system (Ikonomou et al., 2003). Although fungal systems are generally good at production of fungal enzymes, inherent high concentration of proteases and technical limitations in scale-up have limited this system as an expression host to a few targets (Punt et al., 2002).

Posttranslational modifications (PTMs), specifically, nitrogen (*N*-) and oxygen (*O*-) linked glycosylation motifs are of great importance to RP production due to the effect these motifs have on biological and immune activity *in vivo* (Gomord et al., 2010). In order to develop similar glycosylation to native human patterns, which control bio-compatibility and bio-activity of therapeutic protein targets, many therapeutic RPs are produced in mammalian cell lines. Some commonly employed cell lines include baby hamster kidney (Schmidt, 2004), Chinese hamster ovary (CHO) and mouse murine myeloma lines SP2/0 and NS0 (Chu and Robinson, 2001).

These systems have drawbacks of low protein yields and expensive cultivation often requiring complex media containing serum and growth factors, although serum-free media are becoming common (Chu and Robinson, 2001).

Plant based expression systems have emerged as a platform for RP production, specifically therapeutic proteins (Fischer et al., 2004). So-called molecular farming leverages the high biomass yields of crop plants with potentials of creating edible biologics for applications such as vaccination (Yu and Langridge, 2003).

Of the plant based cell systems, unicellular microalgae have recently gained interest as hosts for RP production due to their relatively rapid growth rates in comparison to plant expression systems, favorable transformation turnaround time, ease of containment, scalability, as well as inexpensive cultivation in simple media close in composition to water (Franklin and Mayfield, 2004; Mayfield et al., 2007; Specht et al., 2010). Of the eukaryotic microalgae, the photosynthetic model organism *Chlamydomonas reinhardtii* is largely targeted for genetic manipulation strategies. The genus *Chlamydomonas* has a long history of functional mutation studies as a model photosynthetic organism (Lewin, 1952). Coupled with ease of *C. reinhardtii* transgene insertion (Kindle, 1990) and fully sequenced nuclear, chloroplast, and mitochondrial genomes (Specht et al., 2010), this microalgae is an attractive species for RP technologies. To date, viable RP production has focused on chloroplast transgene expression in *C. reinhardtii* (Bateman and Purton, 2000) as the plastid genome is amenable to efficient homologous recombination, has well described regulation of gene expression (Herrin and Nickelsen, 2004), and has demonstrated protein yields up to 5% of total soluble protein (TSP) (Manuell et al., 2007). In contrast, nuclear gene expression in *C. reinhardtii* has demonstrated comparably low yields of target RPs. Through mutagenesis strategies a strain of *C. reinhardtii* has been engineered which is able to reach 0.2% TSP from nuclear gene expression (Neupert et al., 2009), however, this pales in comparison to chloroplast based strategies. Indeed over the last ten years a great deal of information has been elucidated regarding the regulation of nuclear transgene expression in this organism, such as the requirement for codon optimization to match the ~62% GC content of the *C. reinhardtii* nuclear genome (Ruecker et al., 2008; Shao and Bock, 2008). Optimized promoter sequences and organism specific regulatory elements have also been identified (Schroda et al., 2000), and some reports have demonstrated that nuclear gene expression can be further improved by masking the sequence to be expressed in introns of native genes (Lumbreras et al., 1998; Eichler-Stahlberg et al., 2009).

A common strategy in industrial RP production is targeted secretion of RPs to the periplasmic or extracellular space (Fischer et al., 2004; Schmidt, 2004; Porro et al., 2005). It is considered that

protein localization into the periplasmic space has reduced exposure to protease activity, less inhibitory feedback to gene expression processes, and can allow continuous cultivation in perfusion-style bioreactors. Proteins that are secreted through the ER and Golgi exocytosis pathway are also subjected to PTMs (Kukuruzinska and Lennon, 1998; Doran, 2000; Punt et al., 2002; Porro et al., 2005; Eichler-Stahlberg et al., 2009). Although not confirmed by other reports, a recent report has claimed that *C. reinhardtii* contains sialylated N-glycans similar to mammals, a feature not found in other plant expression systems (Mamedov and Yusibov, 2011). As PTMs are not performed on proteins produced within the chloroplast (Mayfield et al., 2007), the production of nuclear encoded genes targeted for secretion may provide a means of increasing protein production as well as new avenues for RPs depending on target PTM motifs.

In light of developments in synthetic biology, we constructed an expression vector *de novo* from known regulatory elements for *C. reinhardtii* nuclear gene expression (Heitzer and Zschoernig, 2007). Certain markers have been demonstrated with a capacity for secretion outside of the *C. reinhardtii* cell (Ruecker et al., 2008; Shao and Bock, 2008; Eichler-Stahlberg et al., 2009). We postulated that the replacement of predicted secretion signal of the marker luciferase protein from *Gaussia princeps* (*g*Luc) with that of a native *C. reinhardtii* extracellular enzyme carbonic anhydrase 1 (CAH1) (Fukuzawa et al., 1990) would improve transgene localization to the culture media and perhaps RP abundance. This manuscript outlines the combination of synthetic biology, plate level protein abundance assay and RP quantification strategies used to demonstrate robust nuclear gene expression and extracellular localization from transformant *C. reinhardtii* cells.

Materials and methods

Vector construction

To create vector p*g*LUC, the sequence of plasmid pHsp70A/RbcS2-c*g*Luc (Fuhrmann et al., 2004; Heitzer and Zschoernig, 2007) between the restriction endonuclease sites *Sac*I and *Kpn*I was modified *in silico* to contain a codon optimized version of the *Gaussia princeps* luciferase (*g*Luc) marker with C-terminal *Lollium perenne* ice binding protein (Sidebottom et al., 2000; Middleton et al., 2012) (*Lp*IBP) and hexa-histidine tags. Unique restriction digest sites were inserted between each regulatory and gene element. The sequence of *Bgl*II was inserted in the coding region immediately after the *g*Luc predicted N-terminal secretion signal determined by SignalP server (Petersen et al., 2011). The entire cassette was synthesized by GeneArt (Life Technologies) via oligonucleotide annealing. This 1.8 kb fragment was sub-cloned into vector pJR38 (Neupert et al., 2009) between restriction endonuclease sites *Spe*I and *PshA*I to incorporate its paromomycin resistance cassette. DNA was digested with FastDigest® restriction endonucleases, run on a 2% agarose gel at 120 V for 35 min and visualized with SYBR® Safe DNA Gel Stain (Life

technologies). Purified linear fragments were ligated with Quick T4 DNA Ligase (NEB) and transformed into Dh5α *Escherichia coli* cells by heat shock.

Vector p*α*CA*g*LUC was designed to contain the *C. reinhardtii* carbonic anhydrase 1 (Fukuzawa et al., 1990) (CAH1) secretion signal (*α*CA) in place of the predicted secretion signal of the *g*Luc. The *α*CA sequence was amplified by polymerase chain reaction (PCR) using the primers FW_{NdeI} 5' aattcatatgGCGCGTACTGGCGCTCT 3', RV_{BglII} 5' aattagatctAGCCTGCGCGCAGC 3' from a CAH1 containing plasmid template. PCR was cycled with: 95 ˚C for 2:00 min, 35 cycles of 95 ˚C for 30 s, 68 ˚C for 30 s, 72 ˚C for 15 s, followed by 72 ˚C for 8:00 min. Reactions resulted in a single visible band of approximately 60 bp that was processed as above. This fragment and p*g*LUC, were digested with *Nde*I and *Bgl*II and processed through to ligation as above.

Vector pNS*g*LUC was created through amplification of the codon optimized *g*Luc sequence omitting its N-terminal targeting region by PCR using the vector p*g*LUC as template with the primers FW_{NdeI} 5' aattcatatgAAGCCGACGGAGAACAACGA 3', RV_{EcoRV} 5' aattgatatcCGTATCGCCGCCAGCGC 3'. PCR was performed as above with an extension time of 30 s. The amplified DNA fragment was then subcloned into p*g*LUC in place of the *g*Luc fragment as above between the restriction endonuclease sites *Nde*I and *Eco*RV. All vector sequences were confirmed through sequencing by SeqLab (Göttingen, Germany).

C. reinhardtii strains, cultivation, and transformation

Wild type *C. reinhardtii* CC1883 (*Chlamydomonas* resource center, St. Paul, MN, USA) and strain UVM4 (Neupert et al., 2009) were both cultivated for this work. Unless otherwise indicated, strains were cultivated in Tris acetate phosphate (Hutner et al., 1950; Gorman and Levine, 1965) (TAP) or TAP_{agar} media under continuous light regimes of 150 μE $m^{-2}s^{-1}$. Prior to transformation, vectors were linearized with *Spe*I and *Kpn*I restriction endonucleases and purified using peqGOLD Gel Extraction Kit (Peqlab) following manufacturer's protocols. Approximately 3 μg of vector DNA was used in each transformation by glass beads conducted as previously described (Kindle, 1990). Transformations were plated on TAP_{agar} with 10 mg L^{-1} paromomycin under continuous light for seven days until antibiotic resistant colonies were visible. Primary transformants were picked from plate and transferred to a 15 cm plate of the same media so that each colony was placed in an orientation allowing 96 independent colonies per plate (Fig. 2A-C). Replicate plate generation was performed with common 96 element stamping tools. Generally, colonies were allowed to grow for at least one week prior to assay or replicate plating to allow appropriate biomass accumulation.

Bioluminescence assay

Assay of *g*Luc marker protein expression was conducted using buffers and coelenterazine at concentrations previously described (Shao and Bock, 2008). To conduct TAP_{agar} plate level luminescence assessment, three replicate plates of both CC1883 and UVM4 transformant libraries for each vector were generated. Each plate was left in the dark for at least 5 min to prevent autofluorescence interference. Dark adapted colonies were covered with 0.01 mM coelenterazine in assay buffer (Shao and Bock, 2008) and visualized for 1 min in the FUSION FX7™ bioluminescence imaging camera (Peqlab) with native software. Intracellular expression could be similarly visualized using 0.1 mM coelenterazine solution with 5 min exposure. Images were compared for each replicate plate to determine colony luminescence patterns. Four colonies demonstrating consistent and robust luminescence were isolated from libraries and cultivated separately. After one week of growth, selected transformants were screened as above for luminescence signal on a single plate. The transformant demonstrating the most robust luminescence signal was selected for further analysis. In total, one transformant strain of both UVM4 and CC1883 containing each vector were isolated for further analysis.

Quantification of recombinant protein from media

Liquid cultures of each mutant were cultivated in 100 mL TAP media and allowed to reach the late logarithmic phase ($9.0x10^6$ cells mL^{-1} CC1883, $2.3x10^7$ cells mL^{-1} UVM4). 100 mL of culture was centrifuged at 10,000 g and supernatant decanted. The supernatant (culture medium) was then run through a 0.2 μm syringe filter to remove cell debris and frozen in liquid nitrogen. Media was subjected to Lowry assay (Bio-Rad, CA, USA) using lysozyme and BSA as standards, to estimate total extracellular protein. Opaque 96 well micro titer plates were set in a dilution series with a standard commercially available *Gaussia* luciferase (PJK Shop, Germany). The recombinant luciferase was resuspended to a concentration of 1 mg mL^{-1} in distilled water and then diluted to 0.01 mg mL^{-1} in TAP media prior to assay. Dilutions in micro titer plates were conducted with luciferase assay buffer (Shao and Bock, 2008). Isolated culture media was thawed on ice and pipetted in dilution series in the same manner as recombinant standard. 0.01 mM coelenterazine in assay buffer was pipetted into each lane using a multi-channel pipette and samples were visualized in the FusionFX camera with 30 s exposure times. Relative luminescence was quantified using Bio1d software (Vilber Lourmat). Recombinant *g*Luc of 0.01 mg mL^{-1} (0.49 μM) was set as 100 %. All assays were conducted a minimum of three times.

Confirmation of vector insertion polymerase chain reaction (PCR)

Transformant colonies demonstrating both growth on paromomycin containing media and bioluminescence signals were assumed to contain genes of interest, however, a conformational PCR was also performed. DNA was isolated from strains of interest via the chelex method (Cao et al., 2009) and 1 µl of resulting supernatant was used in PCR with the primers FW 5' AAGCCGACGGAGAACAACGA 3', RV 5' GGCGTCGGTCACCACCT 3'. 1 µL (~5 ng) of p*g*LUC vector DNA was used as a positive control, 1 µl ddH_2O was used as a negative control. PCR was performed as above with 45 s extension time.

SDS-PAGE and Western blot analysis

50 mL of culture media was lyophilized and resuspended in distilled water to a 10x concentration. Samples were centrifuged for 3 min at 5000 x g to remove insoluble fraction. 7 µL of concentrated media was supplemented with the same volume 2x SDS sample buffer (120 mM Tris, pH 6.8, 4 % SDS, 20 % glycerol, 60 mM DTT) prior to loading onto sodium dodecyl sulphate polyacrylamide gel for electrophoresis (SDS-PAGE). Samples were separated by Tris-Glycine-SDS-PAGE and blotted on nitrocellulose membranes (GE Healthcare). Immunodetection of *g*Luc was performed with an anti-*g*Luc antibody (NEB, MA, USA) as described by the supplier using enhanced chemiluminescence (ECL; GE Healthcare). Recombinant *g*Luc (PJK Shop) served as positive control. Coomassie Brilliant Blue was used as equal loading control.

Media was subjected to dot blot analysis for RP quantification. 1 µL of pure culture media isolated as above was dotted in three technical replicates on a nitrocellulose membrane. Standards of recombinant *Gaussia* luciferase produced in *Kluyveromyces lactis* (generously provided by Avidity, CO, USA) were diluted and added to the membrane to protein quantities shown. Immunodetection was performed as above. For intracellular recombinant protein assessment, 8 mL of late logarithmic phase culture from each strain was centrifuged at 5000 x g, supernatant decanted and cells resuspended in 100 µL distilled water before snap freezing in liquid nitrogen. 7 µL of cell lysate were mixed with 2x SDS sample buffer before SDS-PAGE and blotting as above.

Nickel affinity chromatography

50 mL of culture media was lyophilized and resuspended in 5 mL affinity buffer (50 mM sodium phosphate buffer pH 8, 1 mM pefabloc (Roth), no imidazole). An insoluble fraction was left after centrifugation and 0.8 mL of Ni-agarose resin (Qiagen) was mixed with 4 mL of media solution under slow rotation at 4 °C for 60 min. Wash and elution buffers contained 300 mM NaCl. Two

washes of 10 mL containing no imidazole were performed. Elution fractions of 1 mL contained imidazole concentrations of 10, 20, 50, 100, 150, 200, and 250 mM, named E1-7 respectively. 180 µL of each sample was subjected to bioluminescence assay with 20 µL of 0.01 mM coelenterazine as above and recombinant *g*Luc was used as a control. Residual samples were precipitated via chloroform/methanol protein precipitation (Wessel and Flügge, 1984) prior to preparation for SDS-PAGE and Western blot analysis as above.

Results

In this study, three vectors were designed and constructed with the aim of establishing a system capable of efficient expression and secretion of RPs from the nuclear DNA of *Chlamydomonas reinhardtii*. After design and construction, the vectors were transformed into the nuclear genome of *C. reinhardtii* wt strain CC1883 and strain UVM4, which is a cell line characterized by generally increased levels of RP expression (Neupert et al., 2009). A total of 576 paromomycin resistant transformant cell lines were isolated for the wt and 672 for UVM4, respectively. The transformant libraries were maintained on plates for further analysis.

Concept of the vector design

A vector concept was devised that would meet the goal of nuclear transgene expression and secretion as well as demonstrate flexibility for future applications. For this concept, *Gaussia* luciferase was selected as the reporter since it has been previously shown that *g*Luc has the potential for strong bioluminescence and in addition can localize outside of the cell when expressed from the nucleus of *C. reinhardtii* (Ruecker et al., 2008; Shao and Bock, 2008). An existing vector with robust *g*Luc expression from *C. reinhardtii* nuclear transformation (Fuhrmann et al., 2004; Heitzer and Zschoernig, 2007) was used as the sequence template to create the gene expression cassette of the vectors in this study (Figure 3-1).

These vectors were designed to exhibit unique restriction digest sites between each regulatory element, including the predicted N-terminal targeting sequence of *g*Luc, to allow future flexibility in gene expression studies. pNS*g*LUC was designed and constructed to not contain any secretion signal (Figu 1, A), therefore, allowing nuclear gene expression and intracellular accumulation of the recombinant target proteins. p*g*LUC (Figure 3-1B) contains the native *g*Luc secretion signal with the addition of a restriction site after this feature. p*α*CA*g*LUC was created by replacing the N-terminal targeting sequence of the *g*Luc with the *α*CA targeting sequence (Figure 3-1C).

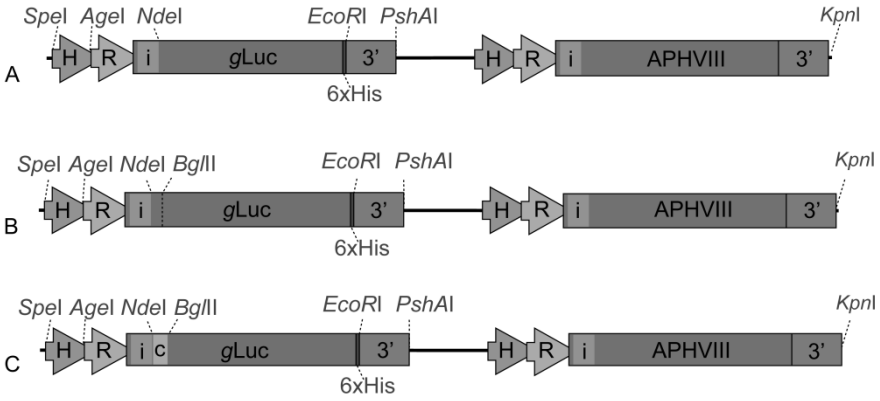

Figure 3-1 Schematic representations of the expression vectors created in this work.
The specific DNA inserts (inserted into vector backbone pJR38) are shown and the unique restriction enzyme cut sites placed between the regulatory elements are indicated.
(A) Vector pNS*g*LUC, comprising the codon optimized *g*Luc (Ruecker et al., 2008) reporter gene with removed secretion signal. (B) Vector p*g*LUC, comprising the full-length codon optimized *g*Luc coding sequence, including the native secretion signal. A *Bgl*II restriction site was added between nucleotides coding for amino acids 17 and 18 to allow further sub-cloning. (C) Vector p*α*CA*g*LUC, comprising the codon optimized *g*Luc coding sequence, in which the native secretion signal of *g*Luc was substituted with the 20 amino acid secretion signal from the *C. reinhardtii* CAH1 enzyme. (H) HSP70A promoter; (R) RBCS2 promoter; (i) RBCS2 intron; (c) Secretion signal of carbonic anhydrase 1; (*g*Luc) Codon optimized *Gaussia* luciferase; (6xHis) Hexa histidine tag; (3') 3' untranslated region of the RBCS2 gene; (APHVIII) Aminoglycoside 3'-phosphotransferase.

Growth characteristics of transformant cell lines

Four transformant lines were selected for each vector and parental strain to determine culture growth characteristics. The selected lines demonstrated similar growth compared to parental strains in mixotrophic conditions. The average culture density at stationary phase for wt CC1883 is approximately $9.1x10^6$ cells mL^{-1} and for UVM4 $2.2x10^7$ cells mL^{-1}. This difference in culture density is related to the cell size of each strain, with CC1883 demonstrating large round cells approximately 10 μm in diameter compared to 4-6 μm for UVM4 in TAP media. No significant difference is observed in culture biomass accumulation between the two strains (not shown), indicating vectors and RP production do not hinder cell viability.

Qualitative bioluminescence plate assays

The functionality of the vectors was assessed via bioluminescence plate assays. No luminescence signal was detectable from parental strains of *C. reinhardtii* in the experimental setup used in this study, either in media samples or at plate level. For all three vectors used, transformants could be identified showing clear bioluminescence signals after coelenterazine addition (Figure 3-2A-C). Bioluminescence signal and intensity varied in-between individual transformants, most likely depending on the level of integrity of the inserted plasmid and/or the locus of insertion. Although variable bioluminescence signals were observed from transformant colonies of all vectors in plate level library screening, we noticed a general tendency for luminescence signals from colonies on p*α*CA*g*LUC library plates (Figure 3-2C) to exhibit stronger luminescence

compared to p*g*LUC counterparts (Figure 3-2B) with equal exposure times. Relative luminesce signals observed from colonies were used to identify the four apparently highest expression lines from each plate, which were then selected for secondary screening (Figure 3-2D-F). Of these four, the transformant for each vector demonstrating the most robust bioluminescence signal was selected for further analysis.

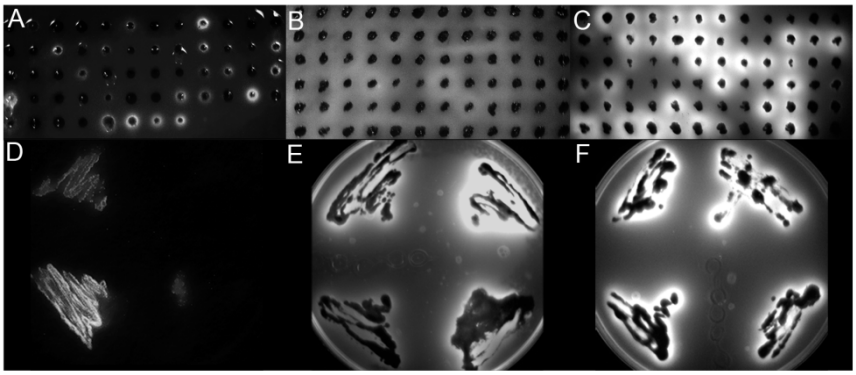

Figure 3-2 Bioluminescence plate assays of cell lines isolated after transformation of the respective expression vectors.
Bioluminescence signals were monitored from transformant colonies growing on solid plates after addition of the *g*Luc substrate coelenterazine.
(A) Intracellular bioluminescence signals of transformants carrying vector pNS*g*LUC. (B) Extracellular bioluminescence signals of transformants carrying vector p*g*LUC. (C) Extracellular bioluminescence signals of transformants carrying vector p*α*CA*g*LUC. (D-F) Relative bioluminescence assessment for selected transformants from plates (A-C), respectively.

When wt or UVM4 cells were transformed with vector pNS*g*LUC, bioluminescence was detected within the cells. Since the native secretion signal from the *Gaussia* luciferase was removed in this vector, this result indicates that the luciferase is expressed within the positive transformants, but not secreted and therefore resides within the cell. When instead vectors p*g*LUC or p*α*CA*g*LUC (including the secretion signal from the *G. princeps* luciferase or the *C. reinhardtii* carbonic anhydrase, respectively) were used for transformation, bioluminescence could be detected within the medium surrounding the transformants. These qualitative results indicated that all three vectors were functional and caused RP expression. Furthermore, the addition of either secretion signal within the amino acid sequence led to effective secretion of the reporter protein into the medium.

Semi-quantitative bioluminescence assay of liquid medium

The bioluminescence plate assays offer the advantage to rapidly screen large numbers of transformants, however, quantification of the individual signal intensities proved to be difficult. To compare the efficiencies of protein secretion mediated by the vectors p*g*LUC or p*α*CA*g*LUC, a semi-quantitative assay with liquid medium was applied. Transformants of both *Chlamydomonas* CC1883 and UVM4 were analyzed. As shown in Figure 3-3, replacing the predicted secretion signal of the *Gaussia* luciferase with the *α*CA N-terminal targeting sequence strongly increased the

detected bioluminescence in the liquid media in both strains by ca. 84% (Figure 3-3), indicating consistently higher protein secretion into the culture medium when the *c*CA secretion signal was used.

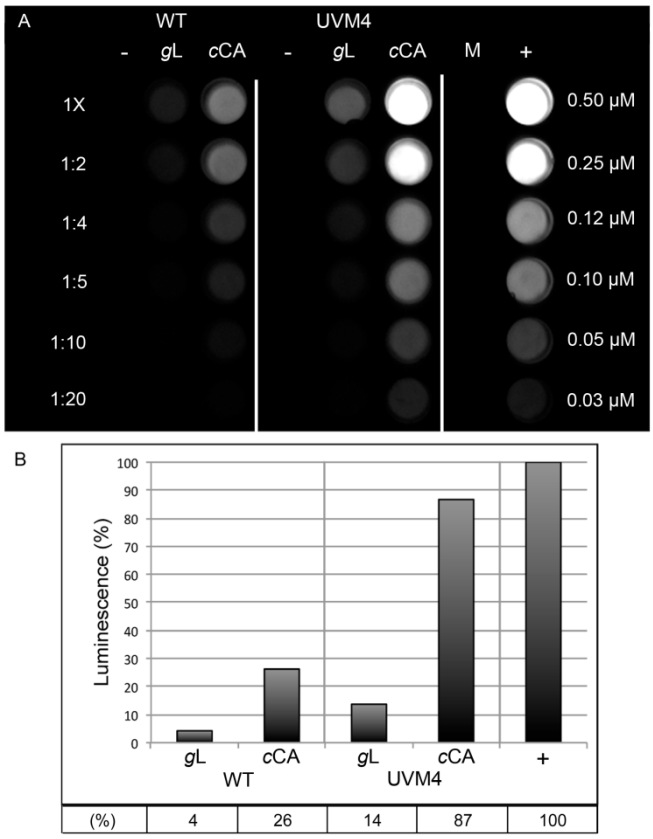

Figure 3-3 Semi-quantitative bioluminescence assays of cell-free supernatant from liquid culture of wt or UVM4 transformants.
Individual cell lines were cultivated, samples harvested from stationary phase cultures, cells removed by centrifugation and the bioluminescence assessed after addition of 0.01 mM coelenterazine.
(A) Dilution assays (1x - 1:20 dilutions) of liquid media of wt and UVM4 transformants are depicted. (B) Graphic representation of the relative luminescence signals, a commercial *g*Luc protein standard (0.01 mg mL^{-1}) was set as 100%. (-) Medium from parental strain; (*g*L) Medium from p*g*LUC transformants; (*c*CA) Medium from p*c*CA*g*LUC transformants. (M) TAP media (+) *g*Luc protein standard.

Recombinant protein quantification

The luciferase-mediated bioluminescence signal can be influenced by many factors, therefore accurate protein quantification via luminescence signal comparison from a standard may be inaccurate. For RP quantification, SDS-PAGE and dot blot Western analyses were applied using an anti-*g*Luc antibody (Figure 3-4). Media samples of logarithmic phase cultures demonstrate total protein contents between 0.5 and 0.8 mg mL^{-1} by Lowry analysis (not shown). A *g*Luc specific signal could be detected slightly below 55 kDa for both parental strains for the p*c*CA*g*LUC transformants in SDS-PAGE Western blots (Figure 3-4A,B). Again, the signal for p*g*LUC appeared to be weaker, especially when wt CC1883 was used as the parental strain (Figure 3-4A).

Dot-blot quantification demonstrated that wt cells expressing the *c*CA fusion protein produced up to ~1.5 mg of RP per liter, whereas UVM4 strains reached values of up to 10 mg L^{-1} without further optimization (Figure 3-4B). UVM4 for both constructs expressed RPs 70% greater than CC1883 (Figures 3-3B and 3-4B). RP accumulation is partially dependent on cell

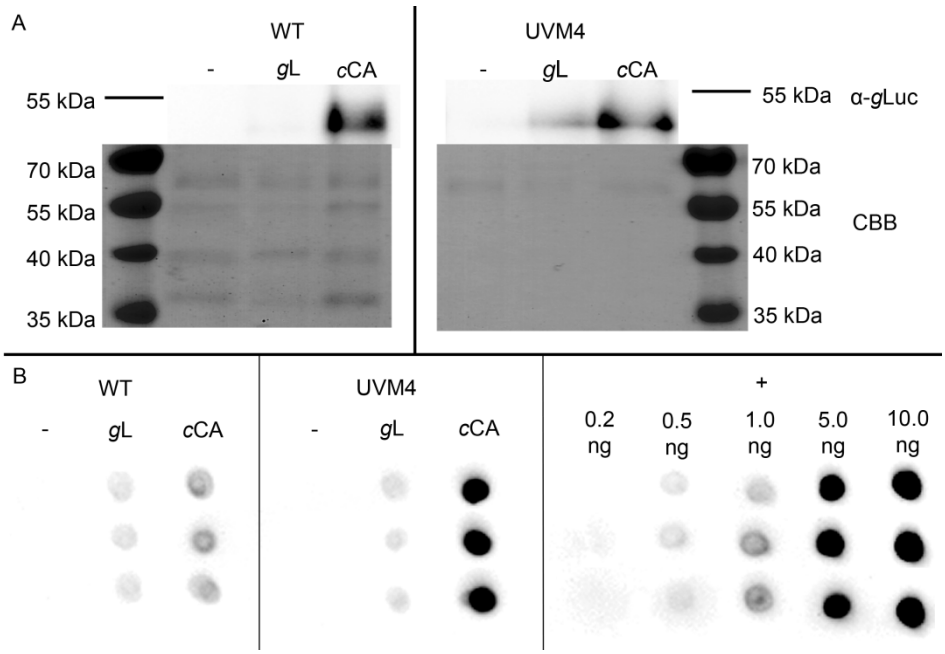

Figure 3-4 Detection (A) and quantification (B) of extracellular RPs in the culture media of individual wt or UVM4 transformant cell lines.
(A) Secreted RPs detected with an anti-gLuc antibody after SDS-PAGE and Western Blots (top panel). Signals specific for gLuc were detected at an apparent molecular weight just below 55 kDa. (CBB) Coomassie Brilliant Blue loading control. (B) Quantification of protein signals (triplicates) by dot blot analysis to assess production and secretion efficiency of wt and UVM4 transformants. Quantification from 1 µL culture medium relative to gLuc standard (+, ng recombinant protein). (-) Represents sample from the respective parental strain; (gL) Represents sample from pgLUC transformants; (cCA) Represents sample from pcCAgLUC transformants.

density (cells mL^{-1}) due to the constitutive HSP70-RBCS2 promoter, however, the greater cell density of UVM4 compared to CC1883 does not entirely account for the observed increased protein accumulation, in agreement with results of previous studies (Neupert et al., 2009). For biotechnological interest, UVM4 far outperformed CC1883 in batch production per unit time accumulating both more cells and RP per liter culture.

Efficiency of the recombinant protein export

Intracellular accumulation of RP within the cells can lead to severe problems for the production host. Therefore, it was important to determine the relative protein export efficiencies for the two vector systems capable of RP secretion (p*g*LUC and p*c*CA*g*LUC). The recombinant *g*Luc protein used in this study (tagged with *Lp*IBP and hexa-his) is predicted to be 31.25 kDa. When cell extracts of wt and UVM4 pNS*g*LUC-transformants were investigated via Western blotting with a commercial anti-gLuc antibody, a clear signal at ~39 kDa was detected, representing intracellular accumulation of the RPs in this cell line (Figure 3-5, NS, black arrows).

The shift of the apparent size detected in the SDS-PAGE compared to the theoretical size could indicate some form of post-translational modification or simply be the consequence of incomplete protein denaturation. In contrast to the pNS*g*LUC-transformants, no signal specific for the RP was detected in cell extracts of p*g*LUC and p*c*CA*g*LUC transformants, indicating

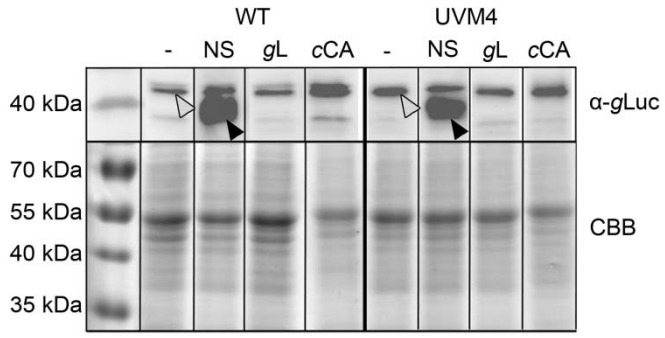

Figure 3-5 Assessment of intracellular accumulation of RPs.
SDS-PAGE and anti-gLuc Western blots were performed with isolated cell extracts from wt and UVM4 transformants of vectors pNSgLUC (NS), pgLUC (gL), pcCAgLUC (cCA). (-) Represents cell extracts of the parental strain (wt or UVM4). Black arrows indicate gLuc specific intracellular signal in pNSgLUC transformants at an apparent molecular weight of ~40 kDa, open arrows indicate unspecific antibody binding detected in all cell extracts. (CBB) Coomassie Brilliant Blue loading control.

efficient targeting and secretion and thus comparably low amounts of residual intracellular RPs (Figure 3-5, *g*L, *c*CA).

Recombinant protein purification by nickel affinity chromatography

For most downstream applications it is desirable to purify the RP of interest after secretion into the culture medium. In this study, the insertion of a hexa-histidine tag at the C-terminus of the secreted protein allowed us to assess the feasibility of nickel affinity chromatography as an initial protein purification step. For this test, a UVM4 transformant of vector p*c*CA*g*LUC was chosen as the highest bioluminescence signals had been detected in the semi-quantitative analyses of the supernatant and the highest quantity of RP was detected for this transformant (Figure 3-3 and 3-4). Interestingly, SDS-PAGE and subsequent Western blotting with the anti-*g*Luc antibody revealed a *g*Luc-specific signal slightly below 55 kDa (Figure 3-6A), therefore significantly higher than the theoretical 31.25 kDa and the 39 kDa signal detected for the intracellular RP in the pNS*g*LUC transformants (Figure 3-5, NS, black arrows). Since it is well established that proteins often can undergo a variety of post-translational modifications (Liu and Howell, 2010), especially glycosylation (Gomord et al., 2010), while traveling through the secretory pathway, this shift in SDS-PAGE mobility of the RP suggest that such a modification did indeed occur, the nature of which currently is not known.

A number of secreted proteins were detectable in the supernatant represented by bands after the SDS-PAGE was stained with Coomassie Brilliant Blue (Figure 3-6B, lane S). The amount of RP was not sufficient to be detected as a single band, but could clearly be detected via Western blotting (Figure 3-6A, lane S). When nickel affinity chromatography was performed with the supernatant, unspecific secreted proteins were lost after elution (Figure 3-6B, E1-E7) and the RP was specifically enriched (Figure 3-6A, E4-E6). Presence of residual RPs in the flow-through

fraction indicates that the amount of RPs in the supernatant was above the binding capacity of the Ni- resin used (Figure 3-6, lane F).

Bioluminescence was used as a control and the patterns in general matched the Western blotting signal intensities (Figure 3-6C). Interestingly, a stronger bioluminescence signal was detected for the flow-through fraction compared to the supernatant fraction, although less RP was present (Figure 3-6A,C, S vs. F), most likely because inhibitory components from the original concentrated media sample in the supernatant were diluted by the elution buffer in the flow-through fraction.

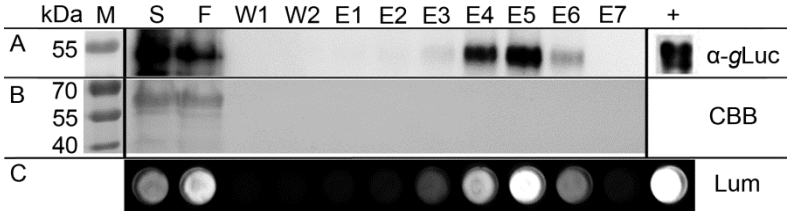

Figure 3-6 Purification of secreted RPs via nickel affinity chromatography.
Medium from a UVM4 p*c*CA*g*LUC transformant culture was subjected to Ni-affinity purification and the fractions were analyzed via SDS-PAGE and Western blots.
(A) RPs detected by anti-*g*Luc Western Blot signals at an apparent molecular weight just below 55 kDa. (B) Visualization of unspecific proteins by SDS-PAGE and Coomassie Brilliant Blue (CBB) staining. (C) Relative bioluminescence signal (Lum) after addition of 0.01 mM coelenterazine. (M) Protein molecular weight marker; (S) supernatant of the transformant culture; (F) flow-through; (W1-2) wash fractions one and two; (E1-7) elution fractions one to seven; (+) recombinant *g*Luc protein standard (runs at an apparent molecular weight of ~25 kDa).

Discussion

A complementary vector system for recombinant protein production, accumulation and secretion

The creation of vector p*g*LUC and its counterparts p*c*CA*g*LUC and pNS*g*LUC, was achieved through the strategy of *in silico* design, oligo annealing DNA synthesis, and subsequent cloning steps. The vector pJR38 (Neupert et al., 2009) was chosen as the donor of both the pBluescript II KS(+) backbone and the APHVIII antibiotic resistance against paromomycin. In these three vectors, unique restriction sites between each regulatory element were designed to promote flexibility of future applications such as promoter characterization, both N- and C-terminal fusion protein strategies, and 3'UTR manipulation. In this work, one successful example for recombinant protein expression, secretion, detection and purification is presented. Although it is possible that the expression of certain target proteins with this new vector system could be prevented by intrinsic inhibitory effects, we suggest that many protein expression studies could benefit from the flexibility of these vectors. pNS*g*LUC is a valuable vector that allows identification of robust protein expression within the cell. In addition, the same cloning flexibility is applicable to the secretion vector p*c*CA*g*LUC. As shown in this work, the combinatory use of both systems can give rise to two sets of transformants, one set efficiently secreting the RP of

interest into the medium, the other efficiently accumulating the RP within the cell, therefore allowing assessment of any potential negative effects of RP production on the host cell. This did not seem to be the case here, as no significant growth deficit was detected for the pNS*g*LUC transformants.

Fast, easy and semi-quantitative initial assessment of expression efficiencies via the Gaussia luciferase reporter

The codon optimized *g*Luc marker has shown its value as a reporter of transgene expression both from intra and extra-cellular targeted constructs before (Ruecker et al., 2008; Shao and Bock, 2008) and has also demonstrated strong bioluminescence in the positive transformants in this work (Figure 3-2). In addition, we added amino acids derived from *Lp*IBP and hexa-histidine residues at the C-terminus for future protein activity studies. The presence of strong bioluminescence is a further proof that *g*Luc is well amenable for fusion constructs. The semi-quantitative luminescence signal produced from the reaction of *g*Luc with coelenterazine allows plate-level screening of transformants for robust protein expression. Bioluminescence assays of replicate plates indicated similar patterning of luminescence signals, allowing identification of robust RP producers without liquid culture scale up (Figure 3-2). Analysis of secreted proteins is simple and can be conducted with dilute amounts of coelenterazine and short exposure times, whereas intracellular expression required higher concentration of this substrate and longer exposure times (Figure 3-2). *g*Luc appeared to be stable in TAP media, the Tris content of this media is likely stabilizing for proteins as we observed no loss of signal or RP degradation during cultivations. The pattern of luminescence seen in plate dilution assay (Figure 3-3) is consistent with RP quantification (Figure 3-4). It should be noted, however, that luciferase activity should not be used as a means of absolute quantification as we observed luminescence activity from *g*Luc can be effected by multiple factors, including production host, buffer composition, and the sensitivity of coelenterazine to light.

Applicability of the vector system in different expression strains and transgene stability

Strain selection is an important aspect of *C. reinhardtii* recombinant gene expression. The new vector constructs were tested in two expression strains. CC1883 was chosen for analysis as this wt strain maintains nitrate reductase capacity and lacks a cell wall, therefore, allows a range of media flexibility. Originally, CC1883 was derived from CC 1690 gr mt+ x CC 1615 cw15 nit2 mt-. In contrast, the heavily mutated UVM4 (Neupert et al., 2009) has been previously described to accumulate higher amounts of intracellular RPs (to 0.2% TSP) and was chosen to determine if this also applied to the extracellular production of RPs with the new vector system. From transformants expressing both intracellular and extracellular targeted *g*Luc, UVM4 demonstrated more positive colony signals in luminescence screening. Bioluminescence signals were generally

more robust from this strain, especially for intracellular protein signals. Compared to wt CC1883, UVM4 demonstrated a shorter time to stationary phase and was more easily cleared from media by centrifugation (not shown). For RP production on an industrial scale it is important that transgene expression is stable for long periods of time. In this respect, all transformants, both from wt and UVM4 parental strains, have been stable for over eight months and continuous cultivation of cells in liquid culture has been performed for four months with no loss of RP secretion activity. Our results indicate that RP production is stable and that UVM4 could be a valuable chassis for secretion based production strategies on larger scale due to its generally increased RP production levels.

Recombinant protein purification

Secretion of RPs is a common strategy in heterologous production concepts (Kukuruzinska and Lennon, 1998; Porro et al., 2005). However, the purification of small amount of proteins from large volumes of media is challenging and a critical step in secretion strategies for laboratory and industrial-scale applications (Ikonomou et al., 2003; Porro et al., 2005). As *C. reinhardtii* can be cultured in water-like media, it has been suggested that purification of RPs from this media may be simplified compared to other systems (Eichler-Stahlberg et al., 2009). Here, Nickel affinity chromatography was conducted with concentrated, soluble fraction and found it possible to isolate the RP of interest from culture media (Figure 3-6). This is a similar finding to that previously described (Eichler-Stahlberg et al., 2009) and demonstrates the potential of this system for other protein targets.

Post translational modifications

Of great importance in the potential use of transgenic microalgae for the production of RPs will be the glycosylation signature of the host species. We observe a shift in gel migration between intra- vs. extracellular targeted RPs indicating PTM, a finding which is not unexpected given the eukaryotic machinery inherent to *C. reinhardtii* (Figure 3-4A, 3-5, and 3-6A). Although two reports currently discuss the potential glycosylation motifs of *C. reinhardtii* proteins (Gomord et al., 2010; Mamedov and Yusibov, 2011), no conclusive study of the PTM signature for this organism has been conducted. Both *N*- and *O*- linked glycosylation states can affect bioactivity of RPs and may also be responsible for immunogenicity (Gomord et al., 2010). Given that many therapeutic proteins require specific glyco-signatures for bioactivity, understanding this mechanism holds great implications for viability of this expression platform. The nuclear genome of *C. reinhardtii* usually does not integrate exogenous DNA via homologous recombination, so is not amenable to the humanization of glycan structures through targeted knockout as in the moss *Physcomitrella patens* (Koprivova et al., 2003; Weise et al., 2007). However, it is not yet clear which glycan

processing capacity is possessed by *C. reinhardtii* as studies of this nature have been limited. Indeed, it will be important to determine the glycosylation potential of RP produced from *C. reinhardtii*, as β1,2 xylose-, α1,3 fucose-, and the Lewis a (Le^a) epitopes typical of plant RPs are characterized as immunogenic (Gomord et al., 2010). The characterization of *C. reinhardtii* PTM signatures is a challenge that will require thorough investigation and was not conducted as part of this study. Understanding these motifs will be aided by the future expression and characterization of multiple RP targets, strategies for which the p*c*CA*g*LUC vector described here will be of value.

The value of microalgal expression platforms and potential applications

Up to 10 mg L^{-1} RP was produced from UVM4 transformed with vector p*c*CA*g*LUC in this work (without further optimization). This is a significantly lower amount of RP protein compared to common fundamental production platforms such as *E. coli* or yeast based expression systems. However, the unique characteristics which apply to microalgae can be of great interest for large-scale RP production concepts as an alternative for other eukaryotic systems. Compared to *Agrobacterium tumefaciens* mediated transformation of plant cells (Yu and Langridge, 2003), transformation and regeneration in *C. reinhardtii* is comparably simple and rapid (Kindle, 1990) with regeneration of transformants occurring in several days. In comparison to mammalian systems, microalgae like *C. reinhardtii* grow well in inexpensive media requiring no serum derived growth factors, use light as an energy source, are generally easy to handle with multiple growth regime possibilities, demonstrate stable nuclear mutations, and are largely amenable to scalability.

Expression and secretion of a RP from a microalgal host presents the possibility of many unique applications given the flexibility of the water-like composition of algal media. It may be possible to secret proteins into this media for direct use if the target RP can function in dilute solutions. A theoretical example of this is the secretion of a monoclonal antibody from *C. reinhardtii* into the culture medium, which then could be used for direct application such as Western blotting. Alternatively, bioremediation strategies could become feasible wherein the secretion of a recombinant, toxin degrading enzyme into effluent water could be conducted in a perfusion-style reactor. Similar studies have demonstrated fungal enzyme expression in microalgae for remediation (Chiaiese et al., 2011) and *C. reinhardtii* has been shown cultivable in effluent water (Kong et al., 2010).

Given the flexibility of optimized vectors generated in this study and the ease of DNA synthesis today, a broad range of target RPs can be envisaged. For proteins, where function is not impacted by glycosylation state, *C. reinhardtii* presents a eukaryotic host which demonstrates robust and stable transformants. Secretion is a means to increase yields and reduce RP degradation while permitting chromatographic purification strategies. Therapeutic protein

production from this system will require further analysis of glycosylation states and thorough investigation of bioactivity from each target as it is developed. The development of an efficient nuclear gene expression system, including ease of transformant identification, and relatively robust RP abundances for *C. reinhardtii*, holds great promise for future large-scale RP expression strategies.

Conclusions

This study presents the design, construction and application of a new, complementary vector system for the expression of recombinant constructs from the nuclear DNA of *C. reinhardtii*. These vectors demonstrate the ability for stable nuclear gene expression of a marker protein both inside the cell and targeted for secretion. Localization into culture media is enhanced by use of the *C. reinhardtii* carbonic anhydrase secretion signal in exchange of the secretion signal found in the native *g*Luc marker protein. The *g*Luc reporter allows fast and easy assessments of the efficiency of RP production, greatly aiding in the identification of robust expression transformants. Our work demonstrates that RP secretion increases with the *c*CA secretion signal consistently by 84% compared to the secretion signal of the *Gaussia* luciferase marker protein alone. In addition, evidence is presented that *C. reinhardtii* strain UVM4 is the preferred host organism for the new vector expression and RP secretion strategy since here, consistently higher amounts of RP were detected. We conclude that the results and new set of vectors generated in this study will benefit future applications of high value RP production from *C. reinhardtii*.

Acknowledgements

The authors would like to acknowledge the CLIB Graduate Cluster Industrial Biotechnology for funding and wish to express their sincere gratitude to Ted Cull of Avidity for providing *g*Luc standard and thoughtful assistance. The authors would also like to thank Prof. R. Bock for providing *C. reinhardtii* strain UVM4.

4 Light-harvesting control under varying carbon supply

4.1 Significance

The results presented in this chapter demonstrate the coordination of a multi-level regulation of light-harvesting with carbon assimilation and the orchestration of short- and long-term responses in a regulatory circuit based on interorganellar communication. Plastid retrograde signals control nuclear promoter activity of photosynthesis associated genes, and control of *NAB1* promoter activity is essential for the adjustment of PSII antenna size upon fluctuating carbon supply. The results of section 4.2 were obtained in collaboration as outlined below, and published in Berger et al. (2014). Section 4.3 compiles unpublished results that complement findings on carbon dependent light-harvesting regulation.

4.2 Publication II

Integration of carbon assimilation modes with photosynthetic light capture in the green alga *Chlamydomonas reinhardtii*

Authors

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published in

Molecular Plant 7:1545-1559.

Author contributions

The experiments were designed by Hanna Berger, Olga Blifernez-Klassen, Lutz Wobbe, and Olaf Kruse.

The experiments were performed by Hanna Berger, Olga Blifernez-Klassen, and Matteo Ballottari. Growth experiments (Figures 4-4, 4-5C,D and S3), creation of a promoter::reporter system and luminescence analysis (Figures 4-2B and S1), pigment analysis (Figures 4-3D and 4-5B), acetate concentration determination (Figure S2), light microscopy (Figure S4), chlorophyll a fluorescence at 77 K (Figure 4-6A,B) and pulse-amplitude-modulation (PAM) fluorescence

measurements (Figure 4-3F,G) were performed by Hanna Berger. With the exception of Figure 4-1B, which was provided by Olga Blifernez-Klassen, all protein analyses via gel electrophoresis and immunoblot detection (Figures 4-1A, 4-2C,D, 4-3A,B, 4-5A and 4-6C) were contributed by Hanna Berger. Quantitative real-time RT-PCR measurements were performed by Hanna Berger (Figure 4-2A) and Olga-Blifernez-Klassen (Figure 4-3C). Matteo Ballottari determined functional photosystem II antenna size using time-resolved fluorescence induction analyses (Figure 4-3E).

Data was analyzed and interpreted by Hanna Berger, Olga Blifernez-Klassen, Lutz Wobbe, and Olaf Kruse.

The Manuscript was written by Hanna Berger, Lutz Wobbe and Olaf Kruse, and edited by Matteo Ballottari and Roberto Bassi.

Abstract

The unicellular green alga *Chlamydomonas reinhardtii* is capable of using organic and inorganic carbon sources simultaneously, which requires the adjustment of photosynthetic activity to the prevailing mode of carbon assimilation. We obtained novel insights into the regulation of light-harvesting at photosystem II (PSII) following altered carbon source availability. In *C. reinhardtii* synthesis of PSII-associated light-harvesting proteins (LHCBMs) is controlled by the cytosolic RNA binding protein NAB1, which represses translation of particular LHCBM isoform transcripts. This mechanism is fine-tuned via regulation of the nuclear *NAB1* promoter, which is activated when linear photosynthetic electron flow is restricted by CO_2 limitation in a photoheterotrophic context. In the wild-type, accumulation of NAB1 reduces the functional PSII antenna size, thus preventing a harmful overexcited state of PSII, as observed in a NAB1-less mutant. We further demonstrate that translation control as a newly identified long-term response to prolonged CO_2 limitation replaces LHCII state transitions as a fast response to PSII over-excitation. Intriguingly, activation of the long-term response is perturbed in state transition mutant *stt7*, suggesting a regulatory link between the long- and short-term response. We depict a regulatory circuit operating on distinct timescales and in different cellular compartments to fine-tune light-harvesting in photoheterotrophic eukaryotes.

Introduction

Photosynthetic acclimation ensures unaffected photosynthetic performance in a constantly changing environment. Light-harvesting is one of its prime targets being modulated on multiple levels with implicated mechanisms operating on different timescales. A sudden rise in light intensity or a drop in CO_2 availability increases excitation pressure at PSII, which has deleterious effects, if not immediately relieved by short-term acclimation mechanisms. Seconds to minutes after the onset of high excitation pressure non-photochemical quenching (NPQ) mechanisms are activated (Allorent et al., 2013). The fast, energy-dependent part of NPQ relies on a reversible switch of light-harvesting complexes from a harvesting into a photoprotective state that is required to dissipate excess excitation energy as heat. This process is regarded as the major photoprotective mechanism in high light (Iwai et al., 2007; Ruban et al., 2007), whereas state transitions represent the predominant fast mechanism that reduces PSII excitation pressure under low light conditions (Rintamäki et al., 1997). An over-reduced plastoquinone pool triggers STT7/STN7-dependent LHCII phosphorylation (Lemeille and Rochaix, 2010; Lemeille et al., 2010) and the subsequent migration of extra or loosely bound trimers (Wientjes et al., 2013a; Drop et al., 2014a) to PSI. Since an enhanced photon absorption capacity at PSI following the state I - state II transition increases cyclic electron flow, this process not only relieves PSII excitation pressure (Bonaventura and Myers, 1969; Murata, 1969), but also adjusts the ATP/NADPH ratio to meet the demands of the Calvin cycle (Bulté et al., 1990; Lemeille and Rochaix, 2010).

State transitions are of particular relevance during the acclimation to varying inorganic and organic carbon supply (Bulté et al., 1990; Johnson and Alric, 2012; Johnson and Alric, 2013; Lucker and Kramer, 2013). Microalgae like *C. reinhardtii* grow photoautotrophically using CO_2 as a carbon and light as an energy source, but in addition reduced carbon sources can be assimilated (Harris, 2009). Being ATP-demanding processes, acetate assimilation and the induction of carbon concentrating mechanisms triggered by inorganic carbon limitation were both shown to induce a transition to state II thereby enhancing ATP-generating cyclic electron flow (Iwai et al., 2007; Lucker and Kramer, 2013). CO_2 limitation reduces the consumption of ATP and NADPH formed within the photosynthetic light reaction leading to an over-reduced photosynthetic electron transport (PET) chain. Previous studies attributed the initial fast reduction in excitation pressure immediately after the onset of CO_2 limitation to state transitions as the underlying mechanism (Palmqvist et al., 1990; Falk and Palmqvist, 1992; Iwai et al., 2007). Reversal of the state II transition during prolonged low CO_2 supply (Iwai et al., 2007), however, indicated that excitation pressure relieve based on state transitions is replaced by alternative mechanisms operating on longer timescales. In accordance with this notion the functional antenna size was shown to be reduced as part of the long-term response to CO_2 limitation in *C. reinhardtii*

(Spalding et al., 1984). Photosynthetic long-term acclimation processes are based on stoichiometric adjustments within the photosynthetic machinery, which require a modulated expression of genes encoding individual subunits. Previous studies analyzing transcriptome changes following CO_2 limitation under low light conditions revealed that the abundance of LHCII (*LHCBM*) transcripts did not change significantly (Yamano et al., 2008). Under such conditions expression of antenna proteins might therefore be regulated post-transcriptionally involving translation control (Wobbe et al., 2008) and this type of control was found in evolutionary diverse photosynthetic organisms (Durnford et al., 2003; Floris et al., 2013; Gutu et al., 2013). In *C. reinhardtii*, the cytosolic RNA binding protein NAB1 represents a key factor controlling the translation of light-harvesting protein encoding transcripts (Mussgnug et al., 2005), that selectively binds to the mRNA of particular *LHCBM* isoforms with a preference for the *LHCBM6* transcript. By sequestrating *LHCBM* mRNA in sub-polysomal ribonucleoprotein complexes, it represses its translation, thereby adjusting the synthesis of LHCII proteins. Given that the demand for light-harvesting proteins in the thylakoid membrane constantly changes in response to environmental cues, cytosolic LHCII translation repression has to be fine-tuned. Two distinct molecular switches in NAB1 were shown to determine its activity and include redox based modification of cysteine residues (Wobbe et al., 2009) besides arginine methylation (Blifernez et al., 2011). Considered that NAB1 represents a key element of the regulatory circuit fine-tuning the PSII light capture, a multi-layer regulation of NAB1-mediated translation control seems reasonable. We investigated the complex regulation of light-harvesting in the photoheterotroph *C. reinhardtii* that follows a switch between carbon assimilation modes and which implicates processes in the nucleus, chloroplast and cytosol. As a key finding, NAB1 was identified as a regulatory hub connecting short- and long-term photoacclimatory responses that control PSII excitation pressure.

Materials and methods

Strains and culture conditions

The wild-types CC849 (cw10; mt-) and CC124 ([137c] mt^-; Chlamydomonas resource center, St. Paul, MN, USA) were used along with the *NAB1* knock out mutant *stm3* derived from CC849 (Mussgnug et al., 2005). For the generation of the *NAB1* overexpressing strain, the *NAB1* gene under control of *PSAD* promoter was introduced into *stm3* (Wobbe et al., 2009). *Stt7* lacking a state transition kinase and the complemented strain *stt7-HA* (Fleischmann et al., 1999; Depège et al., 2003) were obtained from J.D. Rochaix (Geneva, Switzerland).

Media for photoheterotrophic (tris acetate phosphate (TAP) media) and photoautotrophic growth (high salt media, HSM) were prepared as described (Harris, 2009). For phototrophic

short-term induction experiments, TAP medium without acetate was used. For cellulose-dependent photoheterotrophic growth, 0.1% (w/v) carboxy-methyl cellulose (CMC, Carl Roth, 3333.1) was added to HSM and cultures grown under 100 μmol m^{-2} s^{-1} continuous white light without gassing (Blifernez-Klassen et al., 2012). Otherwise, liquid cultures of *C. reinhardtii* were grown in continuous white light at 250 μmol m^{-2} s^{-1} and bubbled with air or CO_2-enriched air (3% (v/v) CO_2). Cultures were always acclimated to the respective starting situation and kept at low cell densities by dilution for at least 48 h prior to the beginning of an experiment. Culture growth was determined as cell density (Z2, Beckman Coulter, Krefeld, Germany) and dry biomass (Bogen et al., 2013). Acetate consumption during growth was determined using an acetate quantification assay (R-Biopharm AG, Darmstadt, Germany) as described (Blifernez-Klassen et al., 2012).

RNA preparation and Quantitative real-time RT-PCR

Total RNA was isolated (Chomczynski and Sacchi, 1987) and quantitative real-time RT-PCR (qRT-PCR) performed as described (Wobbe et al., 2009).

Immunoblotting and densitometrical scanning

Immunoblotting was performed as described (Blifernez et al., 2011) and total cellular protein amount was determined by Lowry assay (Bio-Rad, CA, USA). Immunodetection was performed using enhanced chemiluminescence (ECL; GE Healthcare). Anti-NAB1 antiserum was generated as described (Mussgnug et al., 2005) and anti-LHCBM6/8 (formerly LHCBM4/6) was a kind gift of M. Hippler (Münster, Germany). This antibody recognizes two distinct LHCBM isoforms, namely LHCBM6 and LHCBM8. The LHCII antiserum recognizing all LHCBM isoforms was generated as described (Ferrante et al., 2012). The antibody against PsbA (D1) was obtained from AgriSera (Stockholm, Sweden). For densitometric quantification the software GelAnalyzer 2010a (Lazarsoftware, Hungary) was applied.

Chlorophyll determination and fluorescence analysis

Chlorophyll quantification was carried out as described previously (Wobbe et al., 2009). Room temperature chl *a* fluorescence was measured using Mini-PAM (Walz, Germany). Effective PSII yield (ΦPSII) was determined under growth light conditions and calculated as $(F'_m-F'_t)/F'_m$, maximum PSII yield (F_v/F_m) was recorded after aerobic dark incubation and calculated as $(F_m-F_0)/F_0$. Excitation pressure 1-qP was calculated as described (Maxwell and Johnson, 2000). Fluorescence spectra at 77 K were monitored to determine the relative PSI/PSII emission of cells at a chlorophyll concentration of 10 μg/mL with a Perkin Elmer LS50 spectrometer as described (Kruse et al., 1999) and spectra normalized to the PSII emission maximum at 685 nm.

Relative PSII antenna size was estimated from F_m saturation kinetics ($1/\tau_{2/3}$) in the presence of 10^{-5} M DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea; Ferrante et al. (2012) and kinetics measured with a home-built apparatus. Fluorescence was excited using green LED (peak emission at 520 nm intensity 20 μmol m^{-2} s^{-1}) and detected in the near infrared.

Vector Construction

A 1.55 kb element upstream of the *NAB1* translation start was amplified from *C. reinhardtii* genomic DNA using the sense primer 5'-GGATCCAGTGGCTCAGGGAACCCTAC-3' and anti-sense primer 5'-CATATGTCCCGCGACCCTCCTCGCT-3'. Vector p*c*CA*g*LUC (Lauersen et al., 2013) contains a paromomycine resistance cassette and a codon optimized version of the *Gaussia princeps* luciferase (*g*Luc) combined with a native *C. reinhardtii* secretion signal. To place *g*Luc expression under control of the *NAB1* promoter, the amplified 5' element as well as p*c*CA*g*LUC were digested using XbaI and NdeI FastDigest® restriction endonucleases (Thermo Scientific), purified and ligated as described (Lauersen et al., 2013). The resulting vector contains the *NAB1* promoter element in front of the secretion signal followed by *g*Luc, as confirmed by sequencing (MPIZ DNA core facility on Applied Biosystems; Weiterstadt, Germany).

Transformation and Screening

CC1883 (Chlamydomonas resource center, St. Paul, MN, USA) was used for transformation and reporter assays to allow comparison to strains created previously expressing luciferase under the control of a HSP70/RBCS2 promoter (Lauersen et al., 2013). Transformation by the glass bead method and agar plate based bioluminescence screening using 0.01 mM coelenterazine was performed as published before (Lauersen et al., 2013).

Luminescence Assay

For luminescence quantification, cultures were centrifuged at 1,000 x g for 1 min and resulting supernatants centrifuged again at high speed (10,000 x g) to obtain cell free supernatants. Luminescence was detected by adding 20 μL coelenterazine solution (0.01 mM) to 180 μL supernatant (Lauersen et al., 2013) and recorded for 5 sec in a Sirius-L tube luminometer (Berthold-DS, Pforzheim, Germany)

Statistics

Student's two-tailed t-test for independent samples was applied to statistically evaluate results and significance threshold was set to $p<0.05$. Error bars always represent standard deviations (SD).

Results

The cellular NAB1 level is determined by the prevailing carbon assimilation mode

Green algae like *C. reinhardtii* are photoheterotrophs assimilating organic in addition to inorganic carbon (Harris, 2009). Acetate supply was previously shown to have a repressive effect on photosynthesis including LHCBM expression (Kindle, 1987; Heifetz et al., 2000; Kovács et al., 2000; Humby et al., 2009). The light-harvesting antenna is a prime target of photosynthetic acclimation (Kindle, 1987; Teramoto et al., 2002; Durnford et al., 2003; Rochaix, 2013) and in *C. reinhardtii* translation control of LHCII mRNAs requires the cytosolic translation repressor NAB1 (Mussgnug et al., 2005). Besides its repressor activity, the cellular NAB1 amount is modulated in response to changes in the carbon acquisition mode Blifernez et al., 2011. Immunoblot studies (Figure 4-1A) showed that photoheterotrophic cultivation using air-levels (≈0.04% (v/v)) of CO_2 (-CO_2 throughout the manuscript) and actetate (+Ac) was accompanied by an increased cellular NAB1 amount as compared to photoautotrophic growth with CO_2-enriched air (3% (v/v); +CO_2 throughout the manuscript).

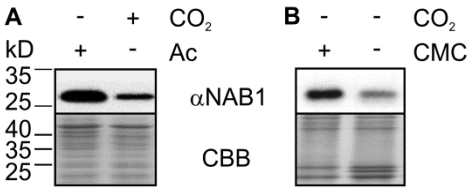

Figure 4-1 The availability and type of carbon source trigger NAB1 accumulation.
(A) Immunodetection of NAB1 in whole cell protein extracts derived from cultures grown in the presence (+) or absence (-) of acetate (Ac). Cultures were either bubbled with air (-CO_2) or CO_2-enriched (3% (v/v)) air (+CO_2). (B) Immunodetection of NAB1 after growth in the presence (+) or absence (-) of carboxymethyl cellulose (CMC) and air-levels of CO_2 (-CO_2). Lower panels (A+B): Coomassie-Brilliant Blue-stain (CBB) serving as a loading control.

C. reinhardtii was recently shown to utilize cellulose as a carbon source (Blifernez-Klassen et al., 2012) and availability of carboxymethyl cellulose (Figure 4-1B, +CMC) in cultures grown under conditions of limiting CO_2 supply (-CO_2) led to a NAB1 accumulation similar to that observed under acetate supply. Although these results do not allow to distinguish between the individual effects of acetate and inorganic carbon supply on NAB1 expression, they indicated that NAB1-mediated translation repression of LHCII mRNAs might be regulated through the overall repressor availability in the cell, which is in turn modulated by the supply of carbon.

CO_2 limitation activates the nuclear NAB1 promoter

Since immunoblot studies (Figure 4-1) showed that carbon source supply strongly impacts NAB1 accumulation, more detailed analyses were conducted to disentangle the individual effects of acetate addition and inorganic carbon availability on NAB1 expression (Figure 4-2). We systematically investigated changes in the transcript level (Figure 4-2A), the modulation of *NAB1*

promoter activity (Figure 4-2B) and protein amounts (Figure 4-2C,D) after adding acetate (Figure 4-2, +Ac) or removing CO_2 (Figure 4-2, -CO_2, white bars) from cultures grown phototrophically and acclimated to CO_2 high levels. Since NAB1 represses *LHCBM6* mRNA translation (Mussgnug et al., 2005), we also monitored both the amount of mRNA and the protein level of *LHCBM6* (Figure 4-2A,C,D). CO_2 limitation increased *NAB1* mRNA levels irrespective of acetate supply (Figure 4-2A, qRT-PCR, *NAB1*) with a twofold (2.2 ±0.4) higher level in cells subjected to acetate and low CO_2 (Figure 4-2A, +Ac, -CO_2) compared to the CO_2-enriched reference culture in minimal medium (Figure 4-2A, -Ac, +CO_2). In contrast *LHCBM6* mRNA levels did not change significantly (Figure 4-2A, *LHCBM6*) when acetate-containing cultures were subjected to inorganic carbon limitation (1.36 ±0.29 /-CO_2 compared to 1.00 ±0.30 /+CO_2).

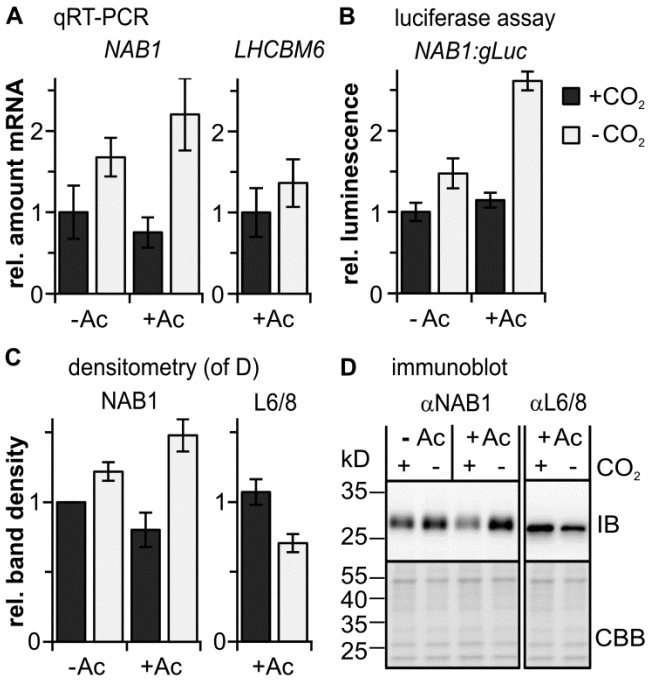

Figure 4-2 Limited CO_2 supply activates the *NAB1* promoter resulting in protein accumulation.
Photoautotrophically cultured cells (3% (v/v) CO_2 in air) expressing the *NAB1*:*g*Luc reporter construct were resuspended in media with (+Ac) or without acetate (-Ac) and bubbled with air (-CO_2) or CO_2-enriched air (3% CO_2) for four hours.
(A) *NAB1* and *LHCBM6* mRNA level assessed by qRT-PCR. (B) Reporter activity in culture supernatants determined by luminescence quantification. (C) Densitometric scanning of NAB1 (αNAB1) and LHCBM6/8 (αL6/8) immunoblot signals (D). Phototrophic cultivation (-Ac/+CO_2) served as the reference condition (set to 1). Error bars represent SD (n=3 for A and B / n=6 for C).

To answer, if the increased mRNA level of NAB1 following CO_2 limitation is based on nuclear promoter activation, reporter constructs (Figure 4-2B) were employed. In these constructs the *NAB1* promoter is fused to a *Gaussia* luciferase (*g*Luc) reporter containing a secretion signal (Lauersen et al., 2013). A wild-type strain was transformed with the nuclear expression vector (Figure S1A) and transformants screened for *g*Luc secretion (Figure S1B), as previously reported (Lauersen et al., 2013). When one of the cell lines showing robust *g*Luc secretion was exposed to CO_2 limitation in the presence or absence of acetate, luciferase activity in culture supernatants (Figure 4-2B and 4-S1D) mirrored mRNA levels (Figure 4-2A), indicating that transcript accumulation following CO_2 limitation is caused by an activation of the *NAB1* promoter.

Comparison of *NAB1* mRNA amounts (Figure 4-2A, *NAB1*) and promoter activities (Figure 4-2B) between cells grown in the presence or absence of acetate when CO_2 supply was high (Figure 4-2, $+CO_2$, ±Ac) indicated that inorganic carbon availability is the major regulator of transcript abundance. Immunoblot studies (Figure 4-2C,D, αNAB1) demonstrated that changes in promoter activity and transcript levels are translated into a modulated protein level. Cells exposed to CO_2 limitation in the presence of acetate showed a 1.5 (±0.1) fold higher level of NAB1 (Figure 4-2C,D, αNAB1, +Ac, $-CO_2$) in comparison to cells grown without acetate but supplied with CO_2 (-Ac, $+CO_2$). The strongest impact of inorganic carbon availability on NAB1 levels, however, was seen when cultures were supplemented with acetate, causing a twofold higher NAB1 amount under low CO_2 conditions (+Ac, $-CO_2$ vs. +Ac, $+CO_2$). As NAB1 was shown to be regulated posttranslationally (Wobbe et al., 2009; Blifernez et al., 2011), a high protein content alone does not necessarily elevate translation repression. Therefore transcript (Figure 4-2A, *LHCBM6*) and protein levels of LHCBM6/8 (Figure 4-2C, D, αL6/8), were analyzed under conditions associated with the lowest and highest NAB1 content (Figure 4-2C,D, +Ac, ±CO_2). Indeed, the accumulation of NAB1 following CO_2 limitation was accompanied by a decline in the LHCBM6/8 protein amount by 34% (Figure 4-2C,D, αL6/8). Together with an unchanged *LHCBM6* mRNA level (Figure 4-2A, LHCBM6) this suggests that NAB1-mediated control of *LHCBM6* mRNA translation is activated in response to CO_2 limitation under photoheterotrophic conditions. The results further demonstrate that LHCBM6 translation repression is triggered by an increased NAB1 availability resulting from promoter activation.

The functional antenna size at PSII is adjusted to the availability of CO_2

To examine the modulation of *LHC* translation repression in further detail, the effect of CO_2 limitation on antenna protein and NAB1 levels was analyzed during photoheterotrophic growth (Figure 4-3). To this end, the parental (Figure 4-3A) and the *NAB1*-free strain (Figure 4-3B) were cultivated in acetate-containing media with continuous low/high or fluctuating CO_2 supply. Cultures were first acclimated to mixotrophic condiditions at low cell density with acetate and high CO_2 provision for 48 h. Then the parental strain and mutant culture were each split into three and grown under high CO_2 concentrations for another twelve hours (Figure 4-3, $t_0/+CO_2$) before bubbling with CO_2-enriched air was either continued ($+CO_2$, black bar) or changed to bubbling with air ($-CO_2$, white bar) besides fluctuating conditions (6 h air, white bar, followed by 6 h of CO_2-enriched air, black bar, $-/+CO_2$). Samples for immunodetection were taken at t_0 and six as well as twelve hours after changing inorganic carbon supply (Figure 4-3A, B, t(h)).

In wild-type cells a reduced availability of CO_2 increased the level of NAB1 already six hours after the onset of limitation (Figure 4-3A, αNAB1, 6 h / -/+CO_2, white bar) and repletion of inorganic carbon fully reversed NAB1 accumulation after six hours (12 h / -/+CO_2, black bar). High NAB1 levels were maintained in cultures provided with air levels of CO_2 for additional six hours (Figure 4-3A, 12 h /-CO_2, white bar), whereas cultures continuously exposed to high CO_2 concentrations showed a low amount of NAB1 (Figure 4-3A, 12 h /+CO_2, black bar). These results are in agreement with the short-term induction pattern (Figure 4-2), where the protein level is lower under mixotrophic conditions with high CO_2 supply than under photoheterotrophy with air levels of CO_2. To test whether the strong difference in NAB1 levels caused a distinct accumulation of the antenna proteins encoded by its main mRNA targets, the LHCBM6/8 amount was analyzed (Figure 4-3A, αL6/8). A low NAB1 level correlated well with a high LHCBM6/8 accumulation after twelve hours of cultivation under high CO_2 conditions (Figure 4-3A, αL6/8, 0 vs. 12 h /+CO_2). The observed LHCII accumulation is not suprising, if it is considered that culture density increases (see below, Figure 4-4) while light availability inside the culture decreases. The inverse correlation of light availability and cellular LHCII amounts is well-described for green algae (Webb and Melis, 1995; Durnford et al., 2003).

In contrast, continuous low CO_2 supply, which increased the level of NAB1, did not cause LHCBM6/8 accumulation (Figure 4-3A, αL6/8, 0 vs. 12 h /-CO_2). As demonstrated previously, the absence of NAB1 (Mussgnug et al., 2005) or a deregulation of its activity (Wobbe et al., 2009; Blifernez et al., 2011) affects the total cellular amount of LHCBM proteins and the accumulation of at least one other LHCBM isoform in addition to LHCBM6. Analysis of the total cellular LHCBM amount (Figure 4-3A, αLHCII) after changing the CO_2 supply revealed that exposure to high CO_2 concentrations causes LHCBM accumulation in wild-type cells (12 h /+CO_2), whereas CO_2 limitation prevents it (12 h /-CO_2). While LHCBM levels increased significantly twelve hours after growth in a CO_2-enriched medium, the protein level of PSII reaction center subunit D1 was unaffected by altered CO_2 availability (Figure 4-3A, αD1). This indicates that the number of PSII reaction centers per cell is modulated to a lower extent than the number of LHCBM proteins, suggesting that CO_2 limitation is accompanied by PSII antenna remodeling.

The strong difference in LHCBM6/8 levels between wild-type cultures continuously provided with either high or low CO_2 levels was not observed for the mutant. Here, LHCBM6/8 and total cellular LHCBM accumulated irrespective of the CO_2 concentration (Figure 4-3B, αL6/8, αLHCII, 12 h /+CO_2 vs. 12 h /-CO_2) demonstrating that the modulation of light-harvesting protein expression in response to CO_2 limitation requires NAB1. The D1 level was relatively unaffected by an altered CO_2 supply in the NAB1-free mutant as well (Figure 4-3A,B, αD1). To

exclude that the increase in LHCBM6 protein levels seen after twelve hours of growth in CO_2-enriched medium for the wild-type is simply based on elevated mRNA amounts, qRT-PCR experiments were performed (Figure 4-3C). Only subtle changes (92 (±6)% /+CO_2 vs. 85 (±4)% /-CO_2) in the *LHCBM6* mRNA level were observed in wild-type cultures following altered CO_2 availability, indicating that translation control rather than *LHCBM6* promoter activity determines protein levels under these conditions. The decrease in *LHCBM6* mRNA levels was stronger in the mutant (Figure 4-3C, grey bars, 81 (±11)% /+CO_2 and 51 (±7)% /-CO_2) which has been reported before (Mussgnug et al., 2005), and explained by a stabilization of *LHCBM6* mRNA in NAB1-containing sub-polysomal complexes that are absent in the mutant. The strong differences in the total cellular LHCBM amount together with an almost unchanged D1 level in both strains following altered CO_2 supply already indicate that the stoichiometry between the PSII core complex and its LHCII antenna is modulated under the conditions examined.

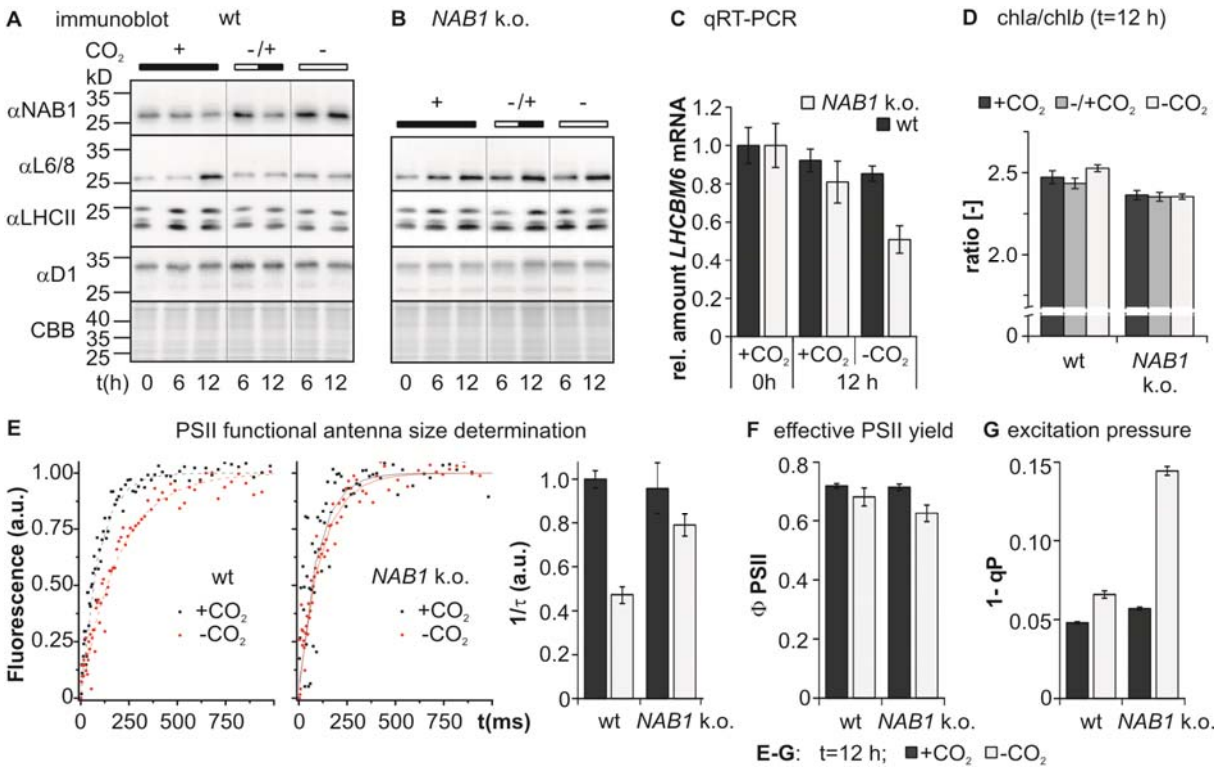

Figure 4-3 Antenna size adjustment during the transition from mixotrophy to photoheterotrophy requires NAB1-mediated translation control.

(A+B) Immunoblot detection of NAB1 (αNAB1), LHCBM isoforms 6 and 8 (αL6/8), all LHCBMs (αLHCII) and D1 protein (αD1) in whole cell samples derived from cultures of the parental strain (wt; A) and the *NAB1* k.o. mutant (B). Cultures acclimated to high CO_2 (3% (v/v)) concentrations (+, t_0) were further exposed to high CO_2 supply (black bar), air levels of CO_2 (-, white bar) or fluctuating CO_2 levels in 6 h intervals (-/+). (C) qRT-PCR quantification of *LHCBM6* transcript levels in parental strain (wt) and *NAB1* k.o. mutant at t_0 and after 12 h of mixotrophic (+CO_2) or photoheterotrophic (-CO_2) growth with level at 0 h set to 1. Indicated are mean values with standard deviations (n=3). (D) Mean chlorophyll *a*/*b* ratios at $t_{12\ h}$ (n=3). (E-G) Chlorophyll *a* fluorescence analyses performed 12 h after changing the CO_2 supply to either low (-CO_2) or high (+CO_2) concentrations. (E) Fluorescence induction kinetics in the presence of DCMU (left panel) and the calculated functional PSII antenna size (1/τ; right panel). (F) Effective PSII quantum yield ΦPSII and (G) excitation pressure. Mean values are shown together with standard deviations (n=9 for E and F; n=3 for G).

To investigate further if the altered NAB1 expression in the wild-type impacts antenna size, the ratio of cellular chlorophyll (Chl) *a* and *b* levels was determined, which exploits the fact that LHC proteins in contrast to other subunits of the photosynthetic apparatus bind Chl *b* besides Chl *a* (Ferrante et al., 2012). In agreement with previous studies (Spalding et al., 1984; Renberg et al., 2010) small differences in Chl *a*/*b* ratios were observed in wild-type cultures grown with distinct CO_2 supply (Figure 4-3D, wt, 2.47 (±0.04) /+CO_2 vs. 2.53 (±0.02) /-CO_2), which indicated that the availability of CO_2 had effects on the antenna protein/reaction center-ratio at both photosystems. It should be noted that in the mutant devoid of NAB1 Chl *a*/*b* ratios remained unchanged in response to altered CO_2 supply (Figure 4-3D, *NAB1* k.o.). This finding indicates that the, albeit small, changes in Chl *a*/*b* seen in wild-type cultures are indeed caused by NAB1-mediated repression of *LHCBM6* translation.

Immunoblot and Chl *a*/*b* data demonstrated that the stoichiometry of LHCBM proteins per reaction center changes in response to inorganic carbon availability. To confirm that these changes indeed alter photon capture capacity at PSII, the functional antenna size was determined in wild-type and mutant after changing the inorganic carbon supply (Figure 4-3E). A strong decrease in the PSII functional antenna size of about 53% (0.47±0.04 for $1/\tau$ and -CO_2 with +CO_2 set to 1) was observed in wt cells upon CO_2 limitation (Figure 4-3E). The same treatment was followed by a more subtle antenna size decrease of 16% in the *NAB1* k.o. mutant (Figure 4-3E, *NAB1* k.o., $1/\tau$: 0.79 (±0.05) /-CO_2 and 0.95 (±0.11) /+CO_2). The inability to adjust the functional antenna size of PSII correlated well with a reduction in the effective PSII yield observed for the NAB1-free mutant upon CO_2 limitation, which indicates an over-reduction of the photosynthetic electron transport chain (Figure 4-3F, ΦPSII, *NAB1* k.o., 0.63 (±0.03) /–CO_2 vs. 0.72 (±0.01) /+CO_2). In contrast to the mutant, the effective PSII yield was relatively unaffected by the availability of CO_2 in wt cultures (Figure 4-3F, wt). Consequently, the increase in excitation pressure (Figure 4-3G, 1-qP) following inorganic carbon limitation was much higher in *NAB1* k.o. cells (2.5 fold) than in cells of the parental strain (1.4 fold).

Acclimation to changes in CO_2 availability in C. reinhardtii requires functional LHC translation control

A functional analysis of the PSII-associated antenna in wt and *NAB1* k.o. cells grown under either high or low CO_2 conditions (Figure 4-3E) demonstrated that antenna remodeling observed after withdrawal of CO_2 in wild-type cells prevented an over-reduction of the photosynthetic electron transport chain and an increased PSII excitation pressure. Our results suggest that the LHCBM6 amount is reduced by NAB1-mediated translation repression under CO_2-limiting conditions and that the extent of repression is fine-tuned via modulation of nuclear *NAB1* promoter activity. It can therefore be hypothesized that growth of the *NAB1* k.o. strain, which is

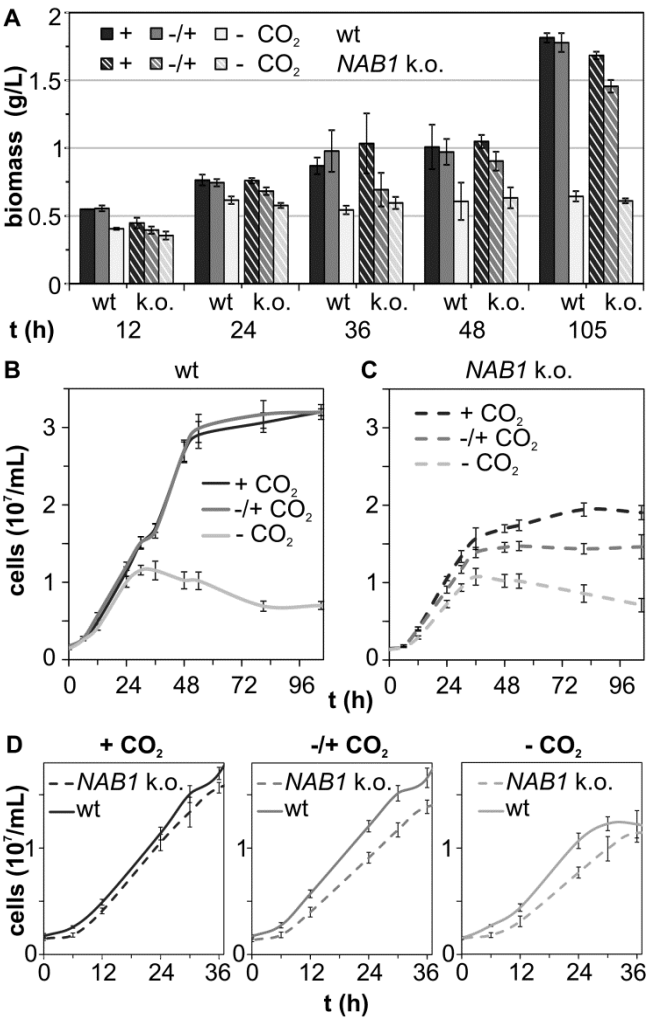

Figure 4-4 The absence of NAB1 reduces growth performance under conditions of fluctuating and low CO_2 supply.
Parental strain (wt; A, B and D) and *NAB1* k.o. mutant (A, C and D) were cultivated as was done for the immunoblot studies shown in Figure 4-3 to analyze the effects of continuous CO_2 supply (black; $+CO_2$), air bubbling (light grey; $-CO_2$) or fluctuating CO_2 provision on the growth performance of both cell lines. Growth was determined as dry biomass (A) and cell density (B-D). (D) Detailed view on the initial growth phase in wt (solid lines) and k.o. mutant (dashed lines) cultures. All data represent mean values ±SD of three biological replicates with triplicate measurements (n=9).

unable to prevent LHCBM6 accumulation after CO_2 limitation, is affected under low or fluctuating inorganic carbon supply. To investigate the anticipated phenotypic consequences in detail, the parental strain was cultivated along with the *NAB1* k.o. mutant as was done for immunoblot studies (Figure 4-3). Acetate consumption (Figure S2) was determined to distinguish between photoheterotrophic, mixotrophic and photoautotrophic growth phases. A complete consumption was reached after 36 h (24 h after gassing change), which is comparable to assimilation rates observed before (Zhang et al., 1999). Growth was monitored as absolute (Figure 4-4A) and relative (Figure S3A,B) biomass accumulation along with cell density (Figure 4-4B-D). In wild-type cultures final biomass yields were almost identical when grown under fluctuating or constant high supply of CO_2 (Figure 4-4A, wt, unshaded bars, $-/+CO_2$ vs. $+CO_2$). In contrast, biomass yields of *NAB1* k.o. cultures were significantly reduced to 89 (±4)% (Figure S3B, 24 h) under fluctuating compared to high CO_2 conditions already within the first photoheterotrophic growth phase (Figure 4-4A, shaded bars, 24 h, 0.68 (±0.03) g/L for $-/+CO_2$ vs. 0.76 (±0.02) g/L for $+CO_2$). At the end of cultivation a reduction to 87 (±3)% (Figure S3B, 105 h) was noted (Figure 4-4A, 105 h, 1.46 (±0.05) g/L for $-/+CO_2$ vs. 1.68 (±0.03) g/L for

CO_2). When CO_2 provision was constantly low both strains stopped accumulating biomass once acetate had been fully consumed (Figure 4-4A, -CO_2, 12 h and 24 h vs. 36 h-105 h). However, after 24 h of growth under low CO_2 supply the mutant accumulated only 76 (±3)% (Figure S3B, 24 h) of the biomass accumulated under high supply (Figure 4-4B, 24 h, 0.58 (±0.02) g/L for -CO_2 vs. 0.76 (±0.02) g/L for +CO_2) while the parental strain reached 82 (±4)% (Figure S3A, 24 h) of the biomass (Figure 4-4A, 24 h, 0.62 (±0.03) g/L for -CO_2 vs. 0.76 (±0.04) g/L for +CO_2).

Cell densities differed more dramatically between both strains (Figure 4-4B, C). For the parental strain, two growth phases could be distinguished. Within the first two days, acetate was consumed (Figure S2) and cells without CO_2 supply (Figure 4-4B, -CO_2) stopped dividing. Cultures supplied with high CO_2 concentrations (Figure 4-4B, +CO_2 and -/+CO_2) rapidly acclimated to the lack of organic carbon after a short lag phase followed by a transition to unperturbed photoautotrophic growth. Complete acetate consumption by the mutant caused a transition to the stationary growth phase independent of the CO_2 supply (Figure 4-4C). Although biomass yields were similar for wild-type and mutant continuously provided with high amounts of CO_2 (Figure 4-4A, +CO_2), final cell densities were strongly decreased in the photoautotrophic phase reaching only 1.90 (±0.11) x 10^7 cells/mL compared to 3.20 (±0.09) x 10^7 cells/mL found in wild-type cultures (Figure 4-4B,C). A stalled cell division together with continued biomass accumulation in the stationary growth phase of the mutant led to morphological changes with mutant cells being larger than their wild-type counterparts (Figure S4, 54 h and 60 h, wt vs. *NAB1* k.o.).

Most importantly, growth of wild-type cultures was insensitive to fluctuating supply of CO_2-enriched air (Figure 4-4B, +CO_2 vs. -/+CO_2), whereas growth of the mutant was clearly impaired under condition (Figure 4-4C, 105 h, 1.90 (±0.11) x10^7 cells/mL for +CO_2 vs. 1.46 (±0.10) x10^7 cells/mL for -/+CO_2). A closer inspection of cell densities during the first two days revealed differences between mutant and parental strain already in the initial phase of the growth experiment (Figure 4-4D). Here, the similar cell morphology allows a direct comparison of the two strains. One day after changing the CO_2 supply, mutant cultures accumulated only 75 (±4)% and 72 (±5)% of the cells found in wild-type cultures under fluctuating and continuous low CO_2 provision, respectively (Figure 4-4D, 24 h, middle and right panel), but only a mild reduction to 92 (±7)% was noted for the high CO_2 condition (Figure 4-4D, 24 h, left panel). This perfectly reflects the NAB1 expression pattern found in wild-type cells with high amounts of NAB1 present under CO_2-limited und low levels prevailing under CO_2-enriched conditions (Figures 4-2 and 4-3) by confirming that the phenotypic difference between parental strain and

mutant is greatest when inorganic carbon is insufficiently provided. In conclusion, a detailed comparative growth analysis of mutant and parental strain disclosed an intriguing new characteristic of the mutant phenotype by demonstrating that acclimation to low and fluctuating CO_2 availability in *C. reinhardtii* requires NAB1.

Fine-tuning of NAB1 promoter activity is crucial for a functional acclimation to fluctuating inorganic carbon supply in C. reinhardtii

The presence of NAB1 is clearly important for an adjustment of the photosynthetic machinery via LHCBM6 expression control under CO_2 limiting and fluctuating conditions. Results obtained with strains expressing a *g*Luc reporter driven by the *NAB1* promoter demonstrated that its activity is modulated in response to changes in CO_2 supply (Figure 4-2B). To confirm that promoter based fine-tuning of NAB1 expression is a central element of the acclimation mechanism, we employed a cell line expressing NAB1 under control of the constitutive *PSAD* promoter (Wobbe et al., 2009). This cell line (*NAB1* oex) is a derivative of the knock out mutant and therefore devoid of endogenous NAB1 expression. In contrast to the wild-type, strain *NAB1* oex did not accumulate NAB1 following CO_2 limitation (Figure 4-5A, αNAB1, 6h, -/+CO_2, -CO_2) and an accumulation of LHCBM6/8 could not be observed twelve hours after exposure to high CO_2 concentrations (Figure 4-5A, αL6/8, 12 h /+CO_2). Again, the D1 level changed little within the course of the experiment (Figure 4-5A, αD1) and similar to what could be observed for the NAB1-free mutant (Figure 4-3D), strain *NAB1* oex displayed only small variations (2.65 (±0.02)/+CO_2, 2.64 (±0.04)/ -/+CO_2 and 2.67 (±0.04)/–CO_2) in the chlorophyll *a*/*b* ratio following exposure to different regimes of CO_2 supply (Figure 4-5B). It should, however, be noted that Chl *a*/*b* ratios in this strain were significantly higher than those determined for mutant and wild-type (Figure 4-3D), indicating that the constitutive availability of high NAB1 levels reduces the number of LHC proteins per reaction center.

Constitutive expression of NAB1 was, however, not sufficient to enable unperturbed growth of *NAB1* oex und fluctuating CO_2 conditions (Figures 4-5C and 4-S3C). Compared to yields under continuous high CO_2 supply, the strain accumulated only 80 (±3)% (Figure S3C) of the biomass (Figure 4-5C, 24 h, 0.47 (±0.02) g/L for -/+CO_2 vs. 0.59 (±0.06) g/L for +CO_2) after 24 h of growth under fluctuating supply and 87 (±2)% (Figure S3C, 105 h) at the end of cultivation (Figure 4-5C, 105 h, 1.60 (±0.03) g/L for -/+CO_2 vs. 1.83 (±0.13) g/L for +CO_2). This impaired biomass accumulation was reflected by cell number reductions. After changing CO_2 supply for 24 h, the culture challenged by fluctuating supply grew to a density of 1.45 (±0.04) x 10^7 cells/mL compared to 1.86 (±0.13) x 10^7 cells/mL found for continuous high supply (Figure 4-5D, 24 h, -/+CO_2 vs. +CO_2).

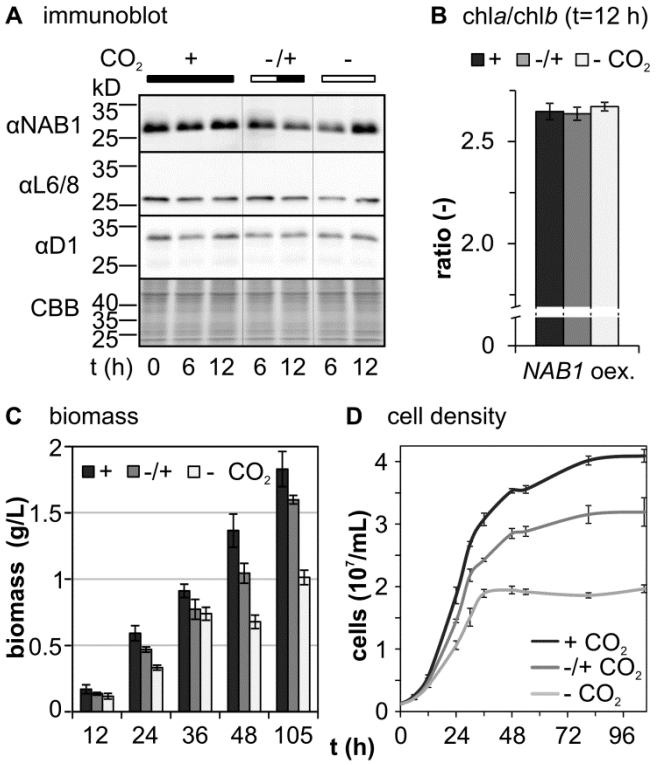

Figure 4-5 A functional NAB1 mediated adjustment of the PSII antenna depends on tight nuclear promoter control.
Cultures of the *NAB1* oex strain were grown for immunodetection (A) and the determination of chl *a*/*b* ratios at $t_{12\,h}$ (B) as described in Figure 4-3. Growth was determined as dry biomass (C) and cell density (D) with data representing means and SD (n=6).

At the end of the cultivation a final cell density reduction by about 22% was noted (Figure 4-5D, 105 h, 3.19 (±0.23) x 10^7 cells/mL for -/+CO_2 vs. 4.09 (±0.10) x10^7 cells/mL for +CO_2).

Overall, fluctuating CO_2 provision, which demands for a fine-tuned NAB1 expression via dynamic promoter modulation, caused a reduced biomass accumulation and cell density in *NAB1* oex cultures (Figure 4-5C,D and 4-S3C, -/+CO_2 vs. +CO_2), whereas growth of the wild-type was relatively unaffected when CO_2 was provided in a fluctuating manner instead of continous provision (Figure 4-4A,B and Figure S3A, -/+CO_2 vs. +CO_2). Thus the introduction of a constitutively expressed NAB1 into the knock out mutant did not restore a wild-type phenotype in regard to flexible antenna size adjustment and growth under conditions of fluctuating CO_2 supply. Faster biomass accumulation and cell division in NAB1 oex compared to wt and NAB1-free mutant might depend on its reduced antenna protein level consistent with the high Chl *a*/*b* ratio. An increased productivity of strains with reduced antenna size has been reported (Polle et al., 2002; Kirst et al., 2012). A direct comparison between *NAB1* oex, mutant and wild-type is of restricted interpretability, considering that only one over-expression line was analysed. Nevertheless, together with *NAB1* transcript and repoter assay data (Figure 4-2) the results suggest that the acclimation to a changing CO_2 environment requires an activity modulation of the *NAB1* promoter in *C. reinhardtii*.

Evidence for a regulatory link between state transitions and post-transcriptional antenna control

A series of experiments clearly demonstrated that the presence of NAB1 is crucial for antenna adjustment in response to CO_2 limitation. This situation is known to cause an over-reduction of the photosynthetic electron transport (PET) chain including the intersystem electron carrier plastoquinone (PQ) and a high excitation pressure at PSII, triggering both state transitions and cyclic electron flow (Iwai et al., 2007; Lucker and Kramer, 2013). To test whether a reduced CO_2 supply combined with acetate addition causes state transitions under the experimental conditions applied throughout the present study, 77K chlorophyll fluorescence measurements were conducted with wild-type cells and the knock out mutant *stt7*, which lacks the STT7 kinase and is hence unable to undergo state transitions (Fleischmann et al., 1999). As a third strain the *STT7*-complemented strain (Depège et al., 2003) was examined.

Low temperature chlorophyll fluorescence measurements were either done in the absence (Figure 4-6A,B, no inh.) or presence (Figure 4-6A,B, +DCMU) of the PET inhibitor DCMU, which prevents a state I - state II transition via oxidation of the PQ-pool (Wollman and Delepelaire, 1984). In the absence of DCMU a strong transition to state II accompanied the switch from inorganic to organic carbon source utilization in wild-type cultures (Figure 4-6A,B upper panels,

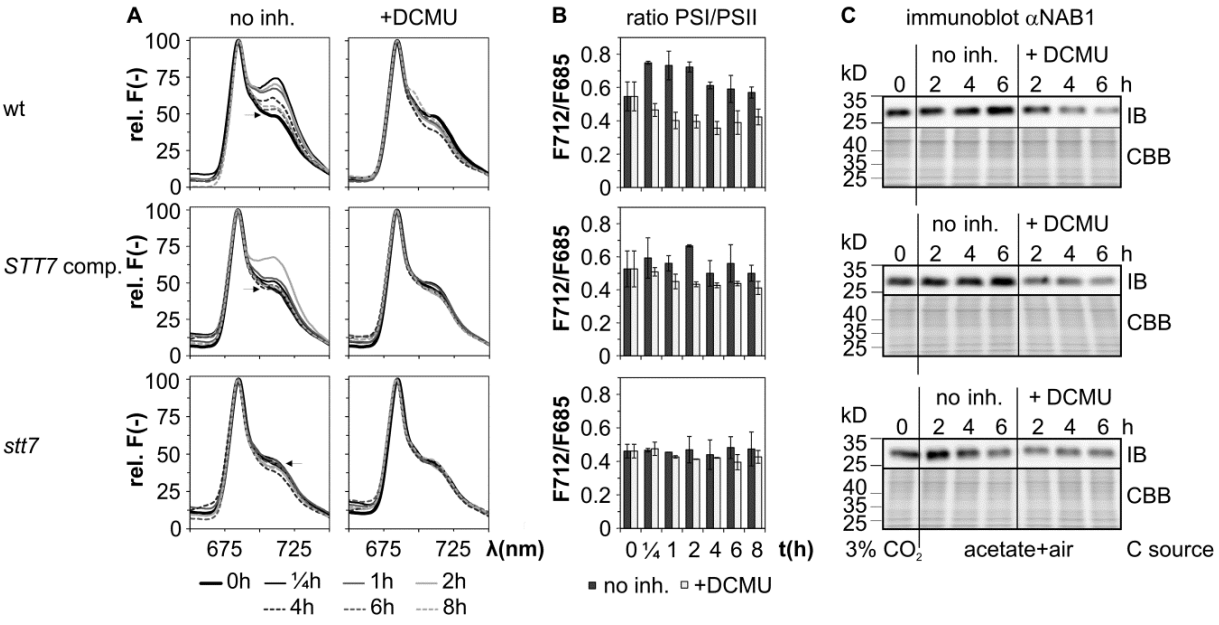

Figure 4-6 NAB1 accumulates under conditions that induce a state II transition and accumulation requires photosynthetic electron transport and the LHCII kinase STT7.

Wild-type cells (upper panels), the state transition kinase deficient mutant *stt7* (lower panels) and the *STT7*-complemented strain (*STT7* comp., second row) were cultivated phototrophically in minimal medium and 3% (v/v) CO_2 prior to acetate addition and CO_2 limitation (air bubbling).

(A) Representative 77 K fluorescence spectra of the time-course experiment performed in the presence (right panels, +DCMU) or absence (left panels, no inh.) of the PET inhibitor DCMU (20 μM). (B) PSI/PSII fluorescence ratios at 77 K determined as F712/F685 in the absence (dark bars, no inh.) or presence of DCMU (light bars, +DCMU). Data represent means and SD of at least two biological replicates. C) Immunodetection of NAB1 in cell lysates of wild-type cells (wt), *stt7* and complemented strain (*STT7* comp) before (0 h, +CO_2) and two to six hours after CO_2 limitation and acetate supply (+Ac), in the presence (+DCMU) or absence of inhibitor (no inh.).

no inh.) was reflected by an increased PSI/PSII fluorescence ratio (Figure 4-6B, wt). This ratio was maintained at a high level within the first two hours before it gradually declined, indicating a transition back to state I despite the presence of acetate and absence of sufficient inorganic carbon. The *STT7*-complemented strain exhibited a reaction similar to the wild-type, though less distinct (Figure 4-6A,B, second row, *STT7* comp.).

In contrast mutant *stt7* did not undergo a transition to state II at all (Figure 4-6A,B lower panels, *stt7*). The slow transition of wt cells back to state I after two hours suggested that state transitions, which are commonly viewed as a short-term acclimation mechanism (Lemeille and Rochaix, 2010) must be replaced by another type of excitation pressure control operating within mid to long timescales.

To get further insights into the potential implication of NAB1-mediated antenna control in the long-term response substituting state transitions only acting on a short timescale, the NAB1 expression pattern was analyzed in strains either containing or lacking kinase STT7 (Figure 4-6C). Similar to what was observed in wild-type cultures (Figures 4-2 and 4-6C, upper panel, wt, no inh.), acetate addition and simultaneous CO_2 limitation triggered an increase in the amount of NAB1 in the *STT7*-complemented strain (Figure 4-6C, second row, *STT7* comp., no inh.). When electron transfer from PSII to plastoquinone was inhibited (Figure 4-6C, wt and *STT7* comp., +DCMU) this treatment not only prevented NAB1 accumulation, but rather caused a decrease of NAB1 levels. DCMU addition had a similar effect on NAB1 expression in the *STT7* k.o. strain (Figure 4-6C, lower panel, *stt7*, +DCMU), which can be anticipated since oxidation of the PQ-pool renders STT7 inactive (Horton and Black, 1980).

Interestingly, the profile of NAB1 accumulation completely differed between *stt7* and the complemented strain or wild-type in the absence of PET inhibitors, when the kinase can be expected to be active (Figure 4-6C, no inh.). Under such conditions *stt7* failed to accumulate NAB1 in response to CO_2 limitation and high organic carbon supply. In summary these observations suggest that a functional state II transition and/or perturbation of PET is a prerequisite for NAB1 accumulation in response to altered carbon availability.

Discussion

Multi-level control of NAB1 mediated LHCII translation repression

The repressor activity of NAB1 was shown to be regulated by two distinct posttranslational modifications (PTMs), including cysteine modification (Wobbe et al., 2009) and arginine methylation (Blifernez et al., 2011). A detailed analysis of NAB1 expression changes following altered organic and inorganic carbon availability (Figures 4-1 and 4-2) demonstrated that the cellular amount of NAB1 is adjusted to the prevailing carbon supply. Inclusion of reporter constructs (Figure 4-2B) and a cell line constitutively expressing high amounts of NAB1 (*NAB1* oex; Figure 4-5) showed that activity modulation of the nuclear *NAB1* promoter is a key mechanism required to fine-tune translation control via NAB1 expression. NAB1 expressed in this strain (Figure 4-5) still contains the cysteine and arginine residues crucial for activity regulation based on PTMs. Because constitutive expression of NAB1, amenable to PTMs, cannot fully complement the knock out growth phenotype seen under conditions of fluctuating CO_2 supply (Figures 4-4 and 4-5), it can be concluded that NAB1 promoter control represents a novel layer of regulation, vital for the precise adjustment of LHCII translation repression.

Translation repression of individual LHCBM isoforms is part of the long-term response to CO_2 limitation

A detailed examination revealed that NAB1 accumulation is enhanced primarily by CO_2 limitation, most prominent along with the addition of acetate as a reduced carbon source. (Figure 4-2). In agreement with previous studies (Teramoto et al., 2002; Yamano et al., 2008), which analyzed the effect of CO_2 limitation on *LHCBM* transcript levels, the removal of CO_2 had only negligible effects on the mRNA level of *LHCBM6* (Figure 4-3C). The amount of LHCBM6/8 protein, however, increased significantly when high CO_2 concentrations were continuously provided in wild-type cultures (Figure 4-3A). Under these conditions cellular NAB1 levels are low (Figure 4-3A), whereas CO_2 limitation triggers NAB1 accumulation accompanied by an unchanged expression of LHCBM6/8 protein. In the *NAB1* k.o. mutant, LHCBM6/8 protein accumulates irrespective of the available CO_2 amount, indicating that the presence of NAB1 is required to prevent an accumulation of LHCBM6/8 under low CO_2 conditions (Figure 4-3B). The effects of NAB1-mediated translation control on LHCBM isoforms 8 (4 in the previous nomenclature; Elrad and Grossman, 2004) and 6 were disentangled in a previous study (Mussgnug et al., 2005), which demonstrated that NAB1 strongly prefers isoform 6 mRNA over that encoding isoform 8, so that the observed NAB1-related changes in LHCBM6/8 expression should mainly reflect a changed amount of LHCBM6 (Figure 4-2A,C,D and Figure 4-3A,C). Analysis of LHCBM accumulation in the NAB1-free mutant (Mussgnug et al., 2005), in cysteine mutants expressing a permanently active version of NAB1 (Wobbe et al., 2009) and after

inhibition of arginine methylation as a PTM maintaining NAB1 in an active state (Blifernez et al., 2011) collectively showed that an altered activity of NAB1-mediated translation repression affects at least one additional isoform besides LHCBM6. Results of the present study further confirm that NAB1, despite of its selectivity, does not exclusively control expression of LHCBM6, nicely explaining why the total cellular amount of distinct LHCBM isoforms is affected by the availability of NAB1, as seen by electrophoretic separation of LHCII-types prior to immunodetection (Figure 4-3A,B, αLHCII).

NAB1 is a regulatory hub connecting short-term and long-term mechanisms of photosynthetic acclimation in C. reinhardtii

The major light-harvesting antenna is a prime target of short- (STR) as well as long-term responses (LTR) to environmental changes increasing PSII excitation pressure and STRs include different types of non-photochemical quenching such as energy-dependent quenching (qE) or state transitions (qT), both reducing PSII excitation pressure, but via distinct mechanisms. LHCII aggregates, similar to those found during qE in plants (Betterle et al., 2009), were observed as an intermediate upon antenna protein detachment during state transitions in green alga (Iwai et al., 2010; Minagawa, 2011) and only a fraction of detached LHCII subsequently bind to PSI (Ünlü et al., 2014). It was therefore suggested, that the main role of state transitions is to decrease the absorption cross-section at PSII rather than maximizing absorption at PSI, which seems to be especially valid for *C. reinhardtii*, since the permanent peripheral antenna of PSI is already much larger than in plant PSI (Minagawa, 2011; Kouril et al., 2012; Ünlü et al., 2014). Among the LTRs are stoichiometric adjustments within the PSII-LHCII complex that reduce excitation pressure by decreasing the absorption cross-section of PSII. This is achieved by a modulated gene expression and, in particular, the regulation of LHCII mRNA translation has been recently demonstrated to represent a crucial mechanism in green alga (Durnford et al., 2003; Mussgnug et al., 2005; Wobbe et al., 2009; Blifernez et al., 2011) and higher plants (Frigerio et al., 2007; Floris et al., 2013). Short- and long-term responses, although operating on different timescales, rely on common plastidic sensors, which indicate photosynthetic imbalances such as the redox-state of intersystem electron carriers or the stroma (Horton and Black, 1980; Pursiheimo et al., 2001; Frigerio et al., 2007; Lemeille et al., 2009).

In the present study the long-term response of *C. reinhardtii* to changes in the availability of organic and inorganic carbon was analyzed with emphasis on the implication of NAB1-mediated translation control. The addition of acetate to *C. reinhardtii* cultures along with CO_2 limitation caused an accumulation of NAB1 (Figures 4-2, 4-3 and 4-6). CO_2 limitation slows down carbon fixation in the Calvin cycle, which attenuates the re-oxidation of NADPH eventually leading to

an over-reduction of the photosynthetic electron transport chain (Lucker and Kramer, 2013) and hence increased PSII excitation pressure (Figure 4-3F,G).

Several previous studies reported that CO_2 limitation triggers a transition from state I to state II in *C. reinhardtii* (Bulté et al., 1990; Iwai et al., 2007; Lucker and Kramer, 2013; Takahashi et al., 2013) and a similar effect was noted after adding acetate to cultures of *C. reinhardtii* (Gans and Rebeille, 1990) or *Chlamydobotrys stellata* (Kovács et al., 2000), which also reduces Calvin cycle activity based on the depletion of plastidic ATP pools via increased cytosolic ATP consumption during the entry of acetate into the glyoxylate cycle.

In agreement with these findings the switch from phototrophic carbon fixation to photoheterotrophic acetate assimilation was accompanied by a state II transition under the experimental conditions used in the present study (Figure 4-6). Despite of the still prevailing low CO_2 supply, the mobile LHCII fraction re-associated with PSII after about two hours, which has also been reported in a previous study (Iwai et al., 2007). In *C. reinhardtii* a low inorganic carbon supply induces a carbon concentrating mechanism (CCM), which is activated within the first three hours after the onset of CO_2 limitation (Renberg et al., 2010). This mechanism effectively increases CO_2 concentrations in the pyrenoid, thus alleviating Calvin cycle inhibition. Within the first hours following inorganic carbon limitation, when the CCM is not fully operational, *C. reinhardtii* cells can be expected to be particular susceptible to photoinhibition. The observed strong state II transition could therefore represent an effective means to prevent photoinhibiton via fast reduction of the PSII absorption cross-section. Although a fully induced CCM effectively concentrates CO_2 at the site of carbon fixation, this mechanism cannot completely compensate the lack of inorganic carbon in air-bubbled cultures, which were provided with CO_2-enriched air before (Falk and Palmqvist, 1992). Consequently, *C. reinhardtii* cells must be equipped with additional acclimation mechanisms that replace state transitions after long-term exposure to insufficient inorganic carbon-supply.

Within four to six hours after changing the supply of carbon NAB1 accumulates in *C. reinhardtii* cells (Figures 4-2, 4-3A and 4-6C) and this elevated expression of NAB1 coincides with a reduction in the level of LHCBM6/8 (Figure 4-2D). A longer exposure of wild-type *C. reinhardtii* cells to a changed carbon supply leads to stoichiometric adjustments within PSII-LHCII complexes (Figure 4-3D) and eventually to an altered functional antenna size (Figure 4-3E), as has been reported before (Spalding et al., 1984). A reduced functional PSII antenna size in wild-type cells under low CO_2, which was less distinct in the *NAB1* k.o. mutant (Figure 4-3E), is a consequence of the elevated NAB1 expression and provides a means to control PSII excitation pressure (Figure 4-3G), when STRs such as state transitions are replaced by LTRs (Figure 4-6).

The *A. thaliana* state transition mutant *stn7*, devoid of a kinase phosphorylating the mobile LHCII faction, was shown to be affected in the LTR to changes in light quality, which were seen as the complete absence of Chl *a*/*b* ratio adjustments typically displayed by wild-type plants under such conditions (Pesaresi et al., 2009). Information on the effects of a *STT7* knock out on LTRs in *C. reinhardtii* is scarce, but results of the present study indicate that an activation of the NAB1-mediated long-term response in this mutant is perturbed (Figure 4-6). During the first two hours, when the transition to state II is most prominent, *stt7* accumulates small amounts of NAB1 (Figure 4-6C), but then fails to increase NAB1 amounts further. This expression course indicates that the physical association of LHCII proteins to PSI, not occurring in the mutant but being of great extent within the first two hours in the wild-type, might not be required to mount the NAB1-mediated LTR. Although *stt7* initially accumulated NAB1, the mutant displayed a NAB1 level significantly below the pre-stress level at the end of the time course, clearly contrasting the expression pattern found in the *STT7*-complemented strain and wild-type (Figure 4-6C).

It is inherently difficult to deduce a direct implication of state transition kinases in the signaling mechanisms underlying the induction of LTRs, since the absence of functional state transitions disrupts redox homeostasis in the chloroplasts, which can be expected to have a great impact on intracellular signaling, that is practically indistinguishable from effects directly caused by the lack of phosphorylating activities. After inhibition of photosynthetic electron transport by DCMU, which creates a leveled, strongly oxidized, redox state in the chloroplast of mutant, complemented strain and wild-type, differences in the NAB1 expression pattern cannot be observed anymore (Figure 4-6C, +DCMU). Therefore observed differences between NAB1 expression patterns in *stt7* and the complemented strain or wild-type, respectively, are only visible when photosynthetic electron flow is active and direct or indirect effects of STT7-inactivation are of relevance.

The following working model can be proposed based on the findings of the present work (Figure 4-7). Under conditions associated to a high carbon-fixing activity of the Calvin cycle, the demand for NADPH produced by linear photosynthetic electron transport is high (Figure 4-7A). A low availability of NAB1 (Figures 4-2C, D, 4-3A and 4-6C) resulting from a decreased nuclear promoter activity (Figure 4-2B) leads to a relaxed LHCII translation control (Figures 4-2A,C,D and 4-3A-C) enabling the assembly of a large PSII-associated antenna (Figure 4-3E) required to provide sufficient energy for PSII photochemistry. Within the long-term response to CO_2 limitation (Figure 4-7B), that increases PSII excitation pressure (Figure 4-3G) by limiting NADPH consumption in the Calvin cycle, the NAB1 promoter is activated (Figure 4-2B) by a plastid retrograde signal that requires photosynthetic electron transport (Figure 4-6C). An increase in the cellular NAB1 amount elevates LHCII translation repression (Figures 4-2C,D and

4-3A-C) thereby reducing the functional PSII antenna size (Figure 4-3E) and relieving PSII excitation pressure (Figure 4-3G). NAB1 accumulates while the transition to state II is being reversed (Figure 4-6) ensuring that short-term acclimation mechanisms reducing PSII excitation pressure are replaced by long-term mechanisms (Figure 4-7C) reducing antenna size based on the stoichiometric adjustment of LHCBM-to-reaction center ratios (Figure 4-3A,B,E). The perturbed induction of NAB1 expression in mutant *stt7*, which lacks the kinase vital for an intact state transition mechanism, suggests a regulatory link between state transitions and LHCII translation control.

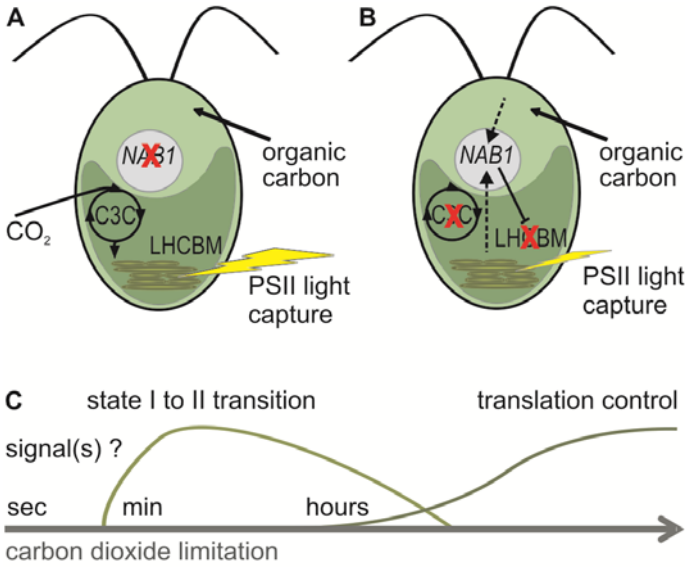

Figure 4-7 NAB1 mediated PSII antenna size control is a long-term response to CO_2 limitation.
(A) A *Chlamydomonas* cell exposed to organic carbon and saturating CO_2 concentrations. The Calvin cycle (C3-cycle, C3C) consumes high amounts of NADPH provided by linear electron transport that requires a large absorption at PSII (yellow flash). Activity of the *NAB1* promoter in the nucleus (grey) is low (red cross) resulting in a reduced availability of the LHCII translation repressor. (B) Limiting CO_2 supply reduces NADPH consumption in the C3-cycle causing increased PSII excitation pressure that has to be relieved by lowering PSII light capture. A retrograde signal (dashed arrow) activates the nuclear *NAB1* promoter to increase the extent of LHCII translation repression in the cytosol. (C) NAB1-mediated translation repression as a long-term response replaces state transitions as a fast mechanism of excitation pressure control.

Overall our data provide important further insights into the multi-level regulation of light-harvesting in lower phototrophic eukaryotes by demonstrating how short- and long-term responses are orchestrated within a sophisticated regulatory circuit based on the intracellular communication between chloroplast, nucleus and cytosol. NAB1 represents an important tool for the future investigation of mechanistic details underlying photosynthetic acclimation in the context of retrograde signaling, especially under consideration of its dual regulation by post-translational modifications (Wobbe et al., 2009; Blifernez et al., 2011) and promoter control as revealed in the present study.

Funding

This work was supported by Deutsche Forschungsgemeinschaft [KR 1586/5-2 to O.K.] and the European Union [ACCLIPHOT to R.B. and SUNBIOPATH No245070 to O.K. and R.B.].

Acknowledgements

The authors would like to thank J.D. Rochaix for providing strains *stt7* and *stt7-HA* and M. Hippler for providing the antibody against LHCBM6/8. We are grateful to the Center for Biotechnology (CeBiTec) at Bielefeld University for access to the Technology Platforms.

4.3 Unpublished results I

Hanna Berger

Author contributions

The experiments shown in this section were designed by Hanna Berger. Hanna Berger performed the experiments, with minor exceptions as indicated, and analyzed as well as interpreted the data. The manuscript was written by Hanna Berger.

4.3.1 Interrelation of state transitions and LHCBM translation control

Introduction

Photosynthesis directly depends on the availability of carbon sources (1.1). Limitation of inorganic carbon and the assimilation of the organic carbon source acetate increase the demand for ATP and lead to an over-reduction of the photosynthetic electron transport chain (Lucker and Kramer, 2013). The interplay of state transitions on short and LHCBM translation control on longer time scales was shown in the present work to adjust photosystem II excitation pressure upon carbon dioxide limitation, most prominently when acetate as organic carbon source was supplied simultaneously (4.2). Intriguingly, besides the observed temporal course of antenna adjustment mechanisms, the regulation of short- and long-term responses appeared to be interdependent, as accumulation of the LHCBM translation repressor NAB1 was impaired in the state transition mutant *stt7* (4.2). Moreover, the *NAB1* knock out mutant was originally described as state transition mutant (*stm3*), unable to reduce photosystem II fluorescence in state II conditions (Kruse et al., 1999; Mussgnug et al., 2005). In this section, the ability of the *NAB1* knock out mutant to perform state transitions and to increase ATP-generating cyclic electron flow was investigated in response to changes in the supply of inorganic and organic carbon. Furthermore, growth of the state transition mutant *stt7*, which is locked in state I, under high, low or fluctuating carbon dioxide supply was determined. The results are interpreted briefly in this section and discussed thoroughly against the background of current knowledge as well as other findings within this work in chapter 6.

Materials and methods

Strains and culture conditions

The wild-type *C. reinhardtii* strain cc849 (cw10; mt-; Chlamydomonas resource center, St. Paul, MN, USA) is the parental strain of the *NAB1* knock out mutant (Mussgnug et al., 2005). *Stt7* lacking a state transition kinase and the complemented strain *stt7-HA* (Fleischmann et al., 1999;

Depège et al., 2003) were kindly provided by J.D. Rochaix (Geneva, Switzerland). Cultures were grown under light intensities of 250 μmol photons m^{-2} s^{-1} with (tris acetate phosphate media; Harris, 2009) or without acetate (high salt media; Sueoka, 1960), and aerated with or without additional 3% (v/v) carbon dioxide. Culture growth was determined as cell density (Z2, Beckman Coulter, Krefeld, Germany) and dry biomass as described in section 4.2.

Fluorescence and electrochromic shift measurements

Measurements were undertaken with or without the addition of DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea). Fluorescence studies were performed at 77 K with a Perkin Elmer LS50 spectrometer to determine the relative PSI/PSII emission of cell cultures with a chlorophyll concentration of 10 μg/mL (Kruse et al., 1999), and spectra were normalized to PSII emission maximum at 685 nm (4.2). Electrochromic shift (ECS) measurements were kindly provided by Matteo Ballottari (University of Verona) using a JTS-10 spectrophotometer with actinic light at 940 μmol m^{-2} s^{-1}. Samples were pre-illuminated at 82 μmol photons m^{-2} s^{-1} for seven minutes.

Results and Discussion

State transitions and cyclic electron flow in the NAB1 knock out mutant under carbon dioxide limitation

The *C. reinhardtii* mutant which lacks the LHCBM translation repressor NAB1 was originally characterized as state transition mutant exhibiting high chlorophyll fluorescence after illumination with PSII light in contrast to wild-type algae (Kruse et al., 1999; Mussgnug et al., 2005). As state II transitions were shown to be a major mechanisms adjusting light-harvesting in the short-term under varying carbon supply in previous (Spalding et al. 1984; Iwai et al. 2007; Lucker and Kramer 2013) and this (4.2) work, the ability of the *NAB1* knock out mutant to perform state transitions upon a switch from inorganic to organic carbon supply, as applied before (4.2), was determined (Figure 4-8). Under these conditions, wild-type cells underwent state transitions, visible as relative increase of PSI fluorescence at 715 nm (Figure 4-8A, wt, no inh.). After two to four hours, state transitions relaxed, consistent with previous observations in this work (Figure 4-6). The PET inhibitor DCMU blocks electron transfer to plastoquinone and in consequence oxidizes the PQ pool, leading to a relaxation of state II. DCMU addition not only prevented a state II transition but led to a decrease of PSI fluorescence (Figure 4-8A, upper panels; +DCMU), indicating that the cells were not in a complete state I under the growth conditions examined, in agreement with previous results presented here (Figure 4-6). Intriguingly, the *NAB1* knock out mutant underwent state transitions as well (Figure 4-8A, *NAB1* k.o.), but initial PSI fluorescence was relatively higher (Figure 4-8, 0 h) and the amplitude of PSI fluorescence change was lower. Also in the mutant strain, DCMU triggered a transition towards

state I, indicating that the mutant is generally able to perform state transitions and is rather in state II under the growth conditions investigated (6.3.3).

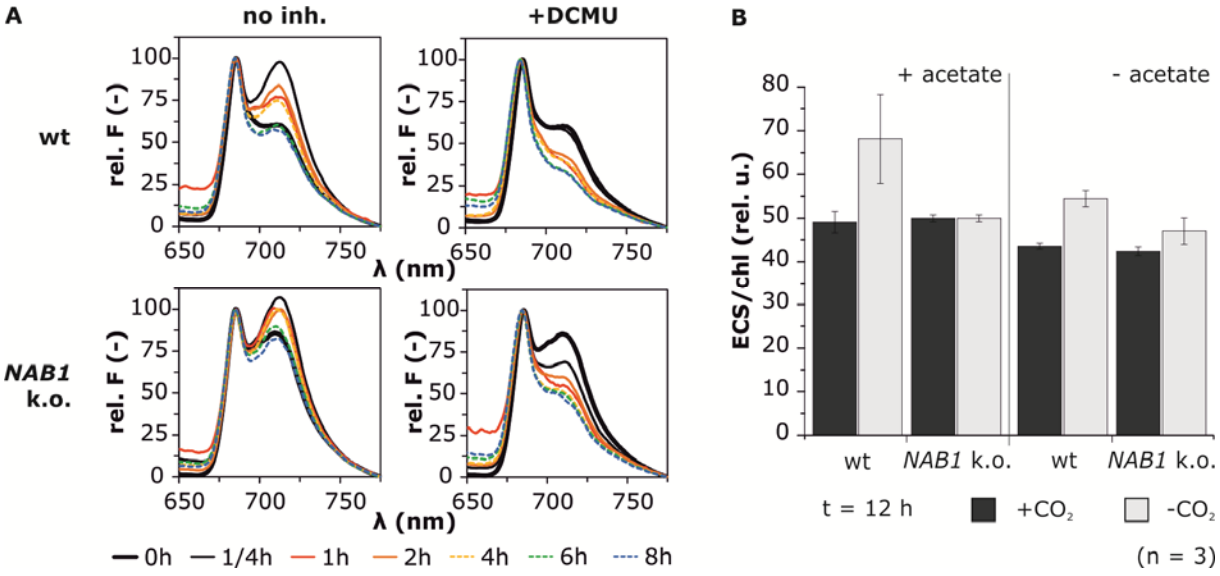

Figure 4-8 State transitions and cyclic electron flow in the *NAB1* knock out mutant.
(A) State transitions of wild-type cells (wt) and the *NAB1* knock out mutant (*NAB1* k.o.) upon a switch from inorganic (3% CO_2) to organic carbon (acetate) supply, measured as time course of 77 K fluorescence spectra in the presence (right panels, +DCMU) or absence (left panels, no inh.) of the PET inhibitor DCMU (20 µmol/L) (4.2).
(B) Cyclic electron flow around PSI determined as electrochromic shift (ECS) (courtesy of Matteo Ballottari). Wild-type and *NAB1* k.o. mutant were grown under 3% (v/v) CO_2 and kept under high carbon dioxide supply (+CO_2) or air levels of carbon dioxide (-CO_2) for twelve hours, either in acetate containing (+acetate) or minimal media (-acetate).

Both carbon dioxide limitation and acetate assimilation raise the demand for ATP (1.1.2). Cyclic electron flow around photosystem I is supposed to increase ATP supply through enhanced photo-phosphorylation under these conditions, and binding of LHCII to PSI in the course of a state II transition could amplify CEF (Lucker and Kramer, 2013; Takahashi et al., 2013; 1.2.2). In order to determine whether cyclic electron flow is increased under the conditions examined in this study and whether the amplitude differs between wild-type alga and the *NAB1* knock out mutant, electrochromic shift (ECS), indirectly reflecting CEF, was measured after twelve hours of high or low carbon dioxide supply, both in the presence and absence of acetate (Figure 4-8B).

Indeed, cyclic electron flow was enhanced in wild-type cells under CO_2 limitation in photoautotrophic and photoheterotrophic growth regimes, and both the relative increase under low CO_2 as well as the overall level was higher when acetate was supplied (Figure 4-8B, wt, - vs. + CO_2 and - vs. + acetate). This indicates that wild-type cells meet the high demand for ATP during carbon dioxide limitation and acetate assimilation through increased photo-phosphorylation. Contrarily, the *NAB1* knock out mutant was unable to enhance cyclic electron flow under low carbon dioxide supply when acetate was present (Figure 4-8B, *NAB1* k.o., + acetate, - vs. + CO_2), while a small increase in CEF could be observed in minimal media (- acetate).

These results obtained in this section are consistent with the depicted importance of NAB1 mediated LHCBM translation repression under carbon dioxide limitation and acetate supply (4.2). While there is little difference between mutant and wild-type under photoautotrophy and high carbon dioxide availability, the observed disability of *NAB1* knock out cells to enhance cyclic electron flow under low CO_2 supply in a photoheterotrophic growth regime could further explain the impaired growth of this strain under these conditions (Figure 4-4), as acetate might be assimilated more slowly due to insufficient ATP generation. Indeed, acetate concentration in the media decreased slightly slower in *NAB1* knock out cell culture (4.2; Figure S2), supporting the considerations about retarded acetate assimilation of this strain.

Growth of the STT7 knock out mutant under fluctuating carbon dioxide supply

The thylakoid kinase STT7 is crucial for LHCII state transitions. While knock out of the homologous kinase in *A. thaliana* impacts long-term regulation of photosynthesis, the *C. reinhardtii* mutant *stt7* has not exhibited significant impairments of photosynthetic acclimation responses on longer time scales and of growth under various physiological conditions, yet (Fleischmann et al., 1999; Depège et al., 2003; Cardol et al., 2009; 1.3.1). In section 4.2 it could be shown that the mutant is impaired in the accumulation of NAB1 upon varying carbon availability. The question arises, whether fluctuating inorganic carbon supply affects the growth of *stt7*. To investigate this, the mutant as well as the complemented strain (*STT7* comp.) were grown in acetate containing media and 3% (v/v) CO_2 before culture gassing was either continued

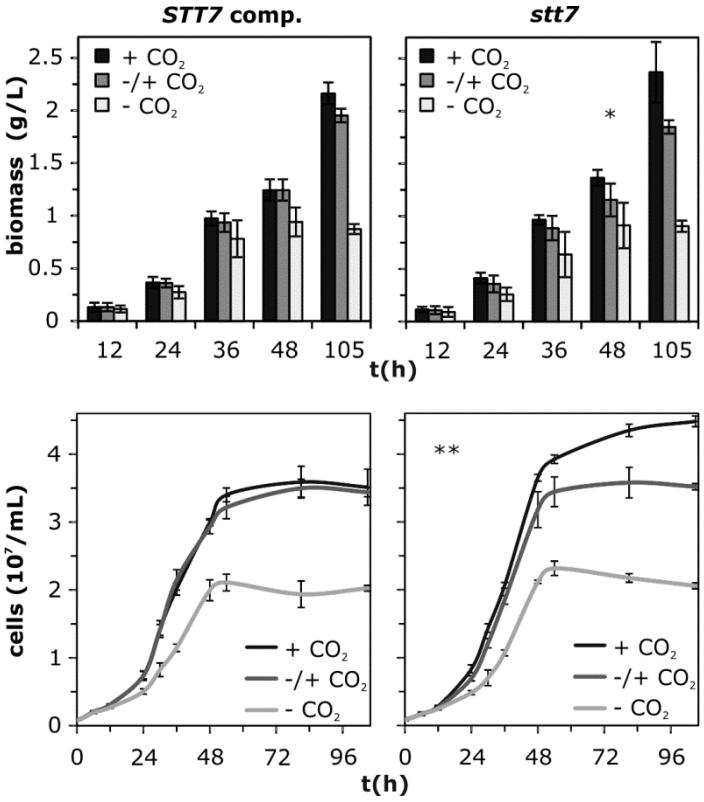

Figure 4-9 Growth performance of *stt7* under varying carbon dioxide supply.
The knock out mutant *stt7* (right panels) and the complemented strain (*STT7* comp. left panels) were acclimated to high CO_2 (3% (v/v)) concentrations and further exposed to high CO_2 supply (+CO_2), air levels of CO_2 (–CO_2), or fluctuating CO_2 levels in 6 h intervals (–/+CO_2). Growth was determined as dry biomass (upper panels) and cell density (lower panels). Significant differences ($p<0.01$) for *stt7* between + and -/+CO_2 (*) from 48 h and (**) from 24 h onwards.

(Figure 4-9; +CO_2) or changed to bubbling with air (-CO_2) or fluctuating carbon dioxide in six hours intervals (-/+CO_2).

Indeed, differences between *stt7* and the control strain emerged. Growth of the complemented strain resembled the wild-type performance (Figure 4-9, *STT7* comp.; compared to Figure 4-4, wt). *STT7* comp. grew equally fast under high and fluctuating carbon dioxide supply. Only in stationary phase a slight reduction of biomass was observed (Figure 4-9, *STT7* comp., 105 h). In contrast, *stt7* showed a slightly decreased growth under fluctuating compared to high carbon dioxide supply (Figure 4-9, *stt7*). This difference was statistically significant ($p<0.01$) after 48 h regarding biomass accumulation (*) and after 24 h regarding cell density (**). Intriguingly, the knock out mutant outperformed the control strain under continuously high CO_2 with a higher cell density and slightly increased biomass (Figure 4-9, +CO_2, *stt7* vs. *STT7* comp.), while under fluctuating carbon dioxide levels biomass accumulation was decreased at similar cell densities (Figure 4-9, -/+CO_2, *stt7* vs. *STT7* comp.), indicating that the mutant cells are smaller under these conditions. There was no significant difference in growth comparing *stt7* and the complemented strain under continuous carbon dioxide limitation, consistent with previous growth experiments (Fleischmann et al., 1999; Depège et al., 2003).

Overall, the results presented in this section confirm the importance of unperturbed light-harvesting regulation under fluctuating carbon availability and the interrelation of short- and long-term regulatory responses (4.2; 6.3.3). The *NAB1* knock out mutant, impaired in LHCBM translation control, was in a permanent state II under growth conditions and was perturbed in enhancing cyclic electron flow under CO_2 limited conditions (Figure 4-8), which likely contributes to the observed growth impairment (Figure 4-4). The state transition mutant *stt7* showed small but significant differences between growth performances under fluctuating compared to high carbon dioxide supply (Figure 4-9), indicating that the mutant cannot completely compensate its deficiency if the environment changes frequently.

4.3.2 Regulation of NAB1 expression

Introduction

A reduction in functional antenna size via LHCBM translation repression efficiently relieved excitation pressure on photosystem II under carbon dioxide limitation (4.2). The increased accumulation of the translation repressor NAB1 emerged as a key component within this response, and the application of a PET inhibitor indicated that signals emerging from the chloroplast control nuclear *NAB1* promoter activity. Consequently, regulatory elements are presumably encoded in the promoter sequence, which allow induction or de-repression of *NAB1*

transcription under carbon dioxide limitation. In order to narrow down DNA sequences essential for this regulation, the *NAB1* promoter::*g*Luc reporter (3.2; 4.2) was employed and truncated promoter elements were tested regarding the ability to confer CO_2 responsiveness. *In silico* analyses were used to identify putative *cis*-regulatory elements (CREs) on the promoter.

Furthermore, high light stress was shown to cause a transient decrease of *LHCBM* mRNA abundance (Durnford et al., 2003; Elrad and Grossman, 2004; 1.2.3). Although transcripts reach pre-stress levels after six two eight hours, LHCII protein levels continuously decrease. It was therefore concluded that post-transcriptional means regulate LHCBM expression under prolonged high light intensities (Durnford et al., 2003). NAB1 mediated translation repression could be part of this control (Erickson et al., 2015). Therefore, NAB1 protein and transcript levels under high light stress were investigated here in order to determine expression of this protein in this condition and to evaluate the role of LHCBM protein synthesis control mediated by NAB1 under high light stress.

Materials and methods

Strains and culture conditions

Strains cc3491 and cc1883 (both cw15; mt-; Chlamydomonas resource center, St. Paul, MN, USA) were used as wild-type *C. reinhardtii*. Cultures were grown photoautotrophically in high salt media (Sueoka, 1960) or with acetate (tris acetate phosphate media; Harris, 2009), bubbled with air or carbon dioxide enriched (3% (v/v)) air. For high light experiments, cells were kept at an optical density of 0.1 at 735 nm ($o.D._{735nm}$, turbidostat) and light intensity was switched from 100 to 1000 µmol photons m^{-2} s^{-1}, mimicking conditions applied in the study of Durnford et al. (2003). Promoter induction experiments with reporter strains (see below) were performed as before (4.2) by incubating the cells in acetate containing media under high or air levels of CO_2 and incident light intensities of 250 µmol photons m^{-2} s^{-1}.

Determination of NAB1 transcription start site

A 5'RACE was performed with modifications according to Scotto-Lavino et al. (2007) to identify the length of the 5'UTR in the *NAB1* gene. RNA was purified from cc1883 cells harvested during late-logarithmic photoheterotrophic growth phase (Chomczynski and Sacchi, 1987). Uncapped RNA was excluded from adapter ligation either by using alkaline phosphatase and tobacco acid pyrophosphatase. The residual RNA, regarded as complete transcripts, was converted into cDNA and amplified using two nested primer pairs (Table 4-1; 5'RACE). PCR products were sequenced (MPIZ DNA core facility on Applied Biosystems; Weiterstadt, Germany). The results of the 5'RACE were validated by performing PCRs (Table 4-1; mapping

of TSS) on gDNA and cDNA of strain cc1883. Products appearing for both templates were considered to be formed within the transcribed region of NAB1 in contrast to those only formed with gDNA as template.

Experimental promoter analysis - Vector construction, strain generation and reporter assay

Three strategies were applied to obtain vectors with truncated *NAB1* promoters (Figure 4-12A). For the 817 and 521 bp fragments, the *g*Luc expression vector containing a 1.55 bp *NAB1* promoter fragment (4.2) was cut with the FastDigest® (Thermo Scientific) restriction endonuclease SpeI (cutting two times) or XbaI and AvrII (cutting one time each). This removed a 5'upstream region, whereas an 817 bp or 521 bp fragment, respectively, relative to translation start remained. After purification, subsequent self-ligation let to the construction of pNabCAgLuc_817 and pNabCAgLuc_521. Fragments containing 398, 283 or 152 bp upstream of translation start were amplified (primers purchased from Sigma Aldrich; Table 4-1), cut with XbaI and NdeI and ligated into the cut and purified vector backbone (4.2). Third, an oligo sequence obtained from annealing Pnab_del_fw and _rv (Table 4-1) was inserted instead of a promoter fragment as a negative control. All vectors were checked by sequencing (MPIZ DNA core facility on Applied Biosystems; Weiterstadt, Germany).

Table 4-1 DNA oligonucleotide sequences.

Name	Sequence 5' → 3'	Comment
Pnab_398_Xbal	AATTTCTAGAGGTTTCCATTGCTC	*NAB1* promoter fragment 398 bp 5' of start-codon
Pnab_283_Xbal	AATTTCTAGAAGGGCTCTGCGT	*NAB1* promoter fragment 285 bp 5' of start-codon
Pnab_152_Xbal	AATTTCTAGAATCGGGGCAATC	*NAB1* promoter fragment 152 bp 5' of start-codon
CAsig_rv	AGGAGTAGAGCGCCAGTACG	reverse primer for Pnab_ forward primers
Pnab_del_fw	CTAGAACTAGTGGATCCCA	deletion of *NAB1* promoter, insert of oligo
Pnab_del_rv	TATGGGATCCACTAGTT	deletion of *NAB1* promoter, insert of oligo
GSP4 RT	TGATGTTGGTCTGGTGCACAAAG	NAB1 coding sequence, rv, 5'RACE
GSP5	GTCTGGTGCACAAAGAGGTC	NAB1 coding sequence, rv, 5'RACE
GSP6	ACTTTACGGTTCCCTGTTGC	NAB1 coding sequence, rv, 5'RACE
N1/A3 II fw	GATATGCGCGAATTCCTGTAG	Adapter specific, 5'RACE
N2/A3 II fw	TCCTGTAGAACGAACACTAGAAGAAA	Adapter specific, 5'RACE
PSP1 rv	CGACCCTCCTCGCTCAGT	*NAB1* promoter -8 bp, mapping 5'UTR
PSP2 fw	CGCCGACCTCGTTACATCT	*NAB1* promoter -100 bp, mapping 5'UTR
PSP3 fw	AATCTCCCGAAGCACACCTT	*NAB1* promoter -147 bp, mapping 5'UTR
PSP4 fw	CAAGCTACGGCACAAATTCA	*NAB1* promoter -265 bp, mapping 5'UTR

Transformation of cc1883, screening and luminescence assay was performed as described in section 4.2. Truncation studies were undertaken with the help of two students, Louise

Brachtvogel and Tatjana Buchholz (Bielefeld University). Three independent cell lines per construct were investigated in three biological and three technical replicates. For all strains analyzed, the insertion of the *NAB1*::*g*Luc fragment was verified by PCR (3.2).

In silico promoter analysis

The databases PLACE (http://www.dna.affrc.go.jp/PLACE; Higo et al., 1999) and PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html; Lescot et al., 2002) were used to search for known *cis*-regulatory element within the 1548 bp fragment upstream of the NAB1 translation start site. Furthermore, motifs which are connected to CO_2-responsiveness as identified in a transcriptome study (Winck et al., 2013a), were taken into account.

Quantitative real-time RT-PCR and immunoblotting

Isolation of total cellular RNA (Chomczynski and Sacchi, 1987) and quantitative real-time RT-PCR (qRT-PCR) using *NAB1* specific primers was performed as described (Wobbe et al., 2009; 4.2). Immunoblot detection of NAB1 protein levels was performed after separation of total cellular protein via 12% tris-glycine SDS-PAGE applying NAB1-specific antiserum (Mussgnug et al., 2005) and enhanced chemiluminescence (GE Healthcare) as before (4.2). Coomassie brilliant blue staining served as loading control.

Results and Discussion

NAB1 expression under high light stress

Post-transcriptional regulation of LHCBM expression was described upon high light treatment in minimal media and low carbon dioxide supply (Durnford et al., 2003; 1.2.3). In order to investigate how NAB1 expression is regulated under this condition and whether NAB1 mediated translation repression could be involved in the decrease of LHCII protein levels, wild-type cells were grown under conditions that mimic those of the study mentioned above, and transcript as well as protein abundance of NAB1 were determined (Figure 4-10). Upon a tenfold increase of

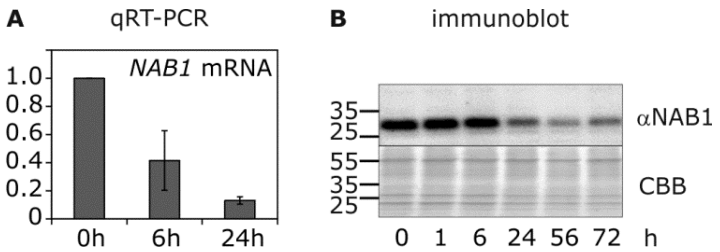

Figure 4-10 High light stress reduces cellular NAB1 mRNA and protein levels.
Wild-type cells were cultured in minimal media with air gassing and were kept at an $o.D._{735nm}$ of 0.1. Light intensity was increased from 100 to 1000 µmol m^{-2} s^{-1}.
(A) *NAB1* transcript levels determined by qRT-PCR with levels at 0 h set to 1. (B) Immunoblot detection of NAB1 protein levels (αNAB1) and coomassie staining (CBB) as loading control.

light intensity to 1000 μmol m^{-2} s^{-1}, *NAB1* mRNA levels decreased to 40% after six hours and below 20% after 24 hours (Figure 4-10A). With a delay, NAB1 protein levels decreased between six and 24 hours and stayed low for at least two more days (Figure 4-10B).

The low NAB1 protein abundance indicates that NAB1 mediated LHCBM translation repression is not the major mechanism reducing light-harvesting proteins levels under prolonged high light. Instead, overall translation could be low due to a global polysome disassembly triggered by reactive oxygen species, as observed under similar conditions (McKim and Durnford, 2006). This study points towards oxidative stress as main determinant of LHCII expression upon high light stress under carbon dioxide limiting conditions. In conclusion, NAB1 mediated translation control operates under more physiological conditions in the absence of severe photoinhibition and oxidative stress.

Identification of the 5'UTR of the NAB1 gene

Activity control of the nuclear *NAB1* promoter is crucial for the regulation of LHCBM translation repression under varying carbon supply (4.2). To characterize the promoter elements in more detail, the transcription start site needs to be determined. To this end, a modified 'new 5'RACE' (Scotto-Lavino et al., 2007) was performed. RNA was harvested from cells in late growth phase. Total RNA samples were treated with alkaline phosphatase and/or tobacco acid pyrophosphatase, allowing discrimination between full-length and broken RNA in subsequent reverse transcription and amplification steps. Fragments obtained specifically in both alkaline phosphatase and tobacco acid pyrophosphatase treated samples were sequenced. Sequencing revealed a 100% identity with a 102 bp fragment of the *NAB1* promoter, merging into *NAB1* coding sequence and flanked by the primers used for amplification No fragment larger than 102 bp could be detected, strongly suggesting that this marks the end of the 5'UTR of the *NAB1* gene.

As a second approach, the approximate length of the 5'UTR was mapped by PCR taking reverse transcribed RNA (cDNA) and genomic DNA (gDNA) as templates. The reverse primer was set to the translation start site, while three forward primers (Table 4-1; mapping 5'UTR) were chosen to bind 100, 147 and 265 bp before translation start (Figure 4-11, ATG).

Amplification of gDNA yielded in visible products for all three forward primers chosen (Figure 4-11, gDNA). In the contrary, cDNA was only amplified using the primer binding 100 bp before translation start (Figure 4-11, cDNA). As apparently primers at position -147 and -265 bp could not bind, this indicates that the end of the 5'UTR of NAB1 is between -100 to -147 bp (Figure 4-11, TSS). This is consistent with the 102 bp fragment found using 5'RACE, as described above.

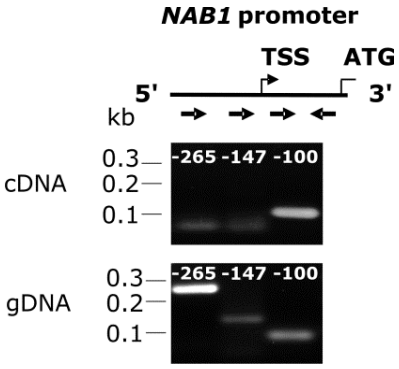

Figure 4-11 Mapping of the 5'UTR of the *NAB1* gene.
Sequences were amplified using gDNA or cDNA as template and *NAB1* promoter specific primers, with forward primers binding 100, 147 and 265 bp before translation start (ATG).

Overall, the results of both approaches are in agreement with a *NAB1* transcription start 102 bp upstream of the translation start codon. The analyses do however not rule out the existence of another transcription start site under different conditions. Transcription from two alternative start sites, one of which representing a TATA-box, was reported for other nuclear *C. reinhardtii* genes before (von Gromoff et al., 2006; Fischer et al., 2009; 1.3.3) Interestingly, a TATA-box and AT-rich region are present in the *NAB1* promoter between -483 to -478 bp and -377 to -358 bp, respectively (Table 4-2), and both could represent sequences of an alternative core promoter.

Regulatory elements on the NAB1 promoter

NAB1 expression is clearly increased under CO_2 limiting conditions, most notably when acetate is present in the culture media, and expression is based on promoter activity (Figure 4-2). In order to narrow down sequences involved in the control of *NAB1* transcription, reporter constructs were created that drive *g*Luc expression from shortened *NAB1* promoter fragments (Figure 4-12). As a negative control, reporter constructs harboring no promoter were introduced into wild-type cells as well (Figure 4-12; 0 bp). For each construct, cells showing luminescence were found amongst 196 transformant colonies screened (Figure 4-12A; luciferase expression: +), and three independent cell lines were analyzed regarding the differential luciferase expression under high (3% (v/v)) and low carbon dioxide supply in acetate containing media.

All cell lines expressing luciferase from a *NAB1* promoter fragment exhibited a higher luminescence under air levels of CO_2 compared to carbon dioxide enriched air (Figure 4-12A; response to CO_2 limitation: +), indicating that the smallest, 152 bp sequence used encodes regulatory elements that confer responsiveness to CO_2 limitation. Importantly, none of the strains with an oligonucleotide (Table 4-1) instead of a *NAB1* promoter sequence showed this response (Figure 4-12A; response to CO_2 limitation: -), confirming that transcriptional and not post-transcriptional regulation is responsible for the observed changes in luminescence.

A time course of luminescence of representative cell lines containing the reporter with the 152 bp *NAB1* promoter fragment and without *NAB1* promoter (0 bp) under low relative to high carbon

dioxide supply is shown in Figure 4-12B. While the relative difference of luciferase expression under CO_2 limitation increased over time for the strain with the 152 bp *NAB1* promoter fragment, the control strain showed similar luminescence under both conditions resulting in a ratio close to 1 between two to eight hours of incubation.

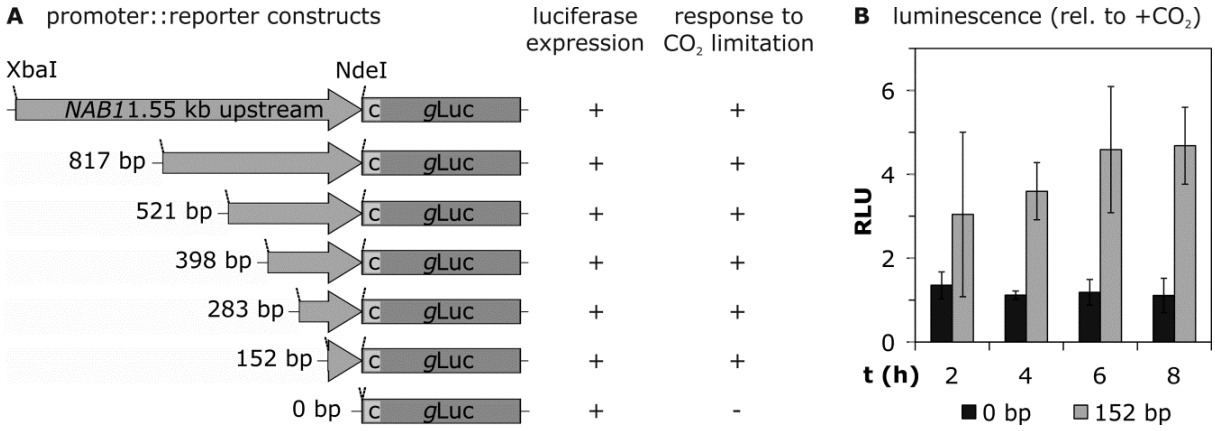

Figure 4-12 Activity of truncated *NAB1* promoter fragments under carbon dioxide limitation.
(A) Luciferase expression and responsiveness to carbon dioxide limitation of truncated *NAB1* promoter::*g*Luc reporter constructs. (B) Luminescence in supernatants of cultures under CO_2 limitation, relative to luminescence under 3% (v/v) CO_2. A representative cell line harboring the reporter with the 152 bp *NAB1* promoter and without *NAB1* promoter (0 bp) is shown; error bars are SD of three biological and three technical replicates (n=9).

Interestingly, the absolute luminescence detected was rather similar in between cell lines harboring a *NAB1* promoter::reporter construct, while this value differed much more for the 0 bp control strains. After eight hours, between 419 and 482 luminescence units per 10^6 cells were detected for the three strains with a 152 bp fragment under CO_2 limitation, while this value ranged from 307 to 1195 luminescence units in the three control strains. This might be explained by a random insertion of the promoter-less luciferase gene behind promoters in the genome ('promoter trap'), which are more or less active under the conditions examined.

Overall, a significant difference ($p<0.01$) was observed in CO_2 responsiveness between cells harboring a 152 bp *NAB1* promoter fragment in front of the *g*Luc gene and those without promoter on the construct. After four hours, the incubation time applied in previous experiments (4.2), the mean ratio of luminescence in air versus +CO_2 of all cell lines and replicates (n=27) was 1.19 (±0.31) for strains without promoter and 3.65 (±1.45) for those with the 152 bp *NAB1* promoter.

To gain further insight into the location of *cis*-regulatory elements on the promoter, the 1.55 kb element was analyzed *in silico* regarding the presence of known *cis*-regulatory elements applying the databases PLACE and PlantCARE as well as information from a recent transcriptome study (Winck et al., 2013a). In the latter publication, transcription factors and regulators which are controlled by the availability of carbon dioxide were identified. The authors could narrow down

ten sequence motifs and respective motif combinations in promoter regions regulated by low carbon dioxide. Intriguingly, six of these motifs are present on the *NAB1* promoter (Table 4-2), however none of them within 152 bp upstream the translation start site. Nevertheless, this study confirms the induction of *NAB1* transcription under CO_2 limitation. NAB1 grouped into a cluster of early responding genes, and mRNA levels were increased by factor 6.9 after one hour, 11.1 after two hours and 3.2 after three hours (Table S3 of Winck et al., 2013a).

Table 4-2 Motifs and putative CREs in the *NAB1* promoter sequence.
CO_2, copper and oxygen, low temp. (temperature): element confers responsiveness to respective factor
put. alt. TSS: core promoter sequence of putative alternative transcription start

Name	Sequence 5' → 3'	Position (bp)	Comment	Source
motif 6	GTTTGCTCAT	-1186 to -1176	CO_2	Winck et al. (2013a)
motif 7	GCCGTTTGCGCAGGTCCTT	-624 to -606	CO_2	Winck et al. (2013a)
motif 4	CGTCTTTGACGTGG	-497 to -484	CO_2	Winck et al. (2013a)
motif 2	TATTAATAAATT	-373 to -362	CO_2	Winck et al. (2013a)
motif 3	AGCAAATAAAGACA	-358 to -345	CO_2	Winck et al. (2013a)
motif 1	AGCATTTGCAGCCGG	-300 to -286	CO_2	Winck et al. (2013a)
curecore	GTAC	-1486; -1373; -1343; -1123; -172; -160	copper and oxygen	PLACE
ltre	CCGAAA or CCGAC	-580; -515; -93; -26	low temp.	PLACE, PlantCARE
TATA-box	TATAAA	-483 to -478	put. alt. TSS	-
AT-rich	TATTTATTAATAAATTAAAA	-377 to -358	put. alt. TSS	-
5'UTR	see appendix, Figure S5	-102 to 0	TSS at -102 bp	this work (4.3.2)

The databases PLACE and PlantCARE focus on regulatory elements identified in vascular plants, but also motifs of *C. reinhardtii* were added. Using these tools, numerous elements can be found on the *NAB1* promoter, and the most relevant, as they were originally detected in *C. reinhardtii* and/or are associated to carbon metabolisms or light-harvesting regulation, are described here.

Four elements responding to low temperatures, but which are also involved in light signaling, are present on the *NAB1* promoter (Table 4-2, ltre); the sequence CCGAAA originally described in in barley (Dunn et al., 1998) at position -580 bp and -515 bp, and CCGAC identified in *A. thaliana* (Kim et al., 2002) and winter *Brassica napus* (Jiang et al., 1996) within the *NAB1* 5'UTR at -93 bp and -26 bp before translation start site. Furthermore, an element that confers responsiveness to copper and oxygen deficiency in *C. reinhardtii* (Quinn et al., 2002; Kropat et al., 2005) is encoded six times on *NAB1* promoter (Table 4-2, curecore).

An enhanced expression of NAB1 during cold periods and hypoxia seems to be reasonable. Low temperatures slow down metabolic reactions such as the Calvin cycle, and oxygen limitation decreases the consumption of reducing equivalents in the mitochondrial electron transport chain

(1.1.1). Both causes an over-reduction of the photosynthetic electron transport chain and increases photosystem II excitation pressure. NAB1 mediated repression of LHCBM protein synthesis could lower the PSII antenna size under cold or hypoxic conditions and relieve the pressure (6.2.1).

Two enhancer elements with the consensus motif GANTTNC, the binding sites site of the transcription factor LCR1 to the *CAH1* promoter, are crucial for expression induction of the carbonic anhydrase 1 under carbon dioxide limitation (Kucho et al., 2003; Yoshioka et al., 2004). The consensus sequence is found five times on the *NAB1* promoter, but exclusively on reverse strand, indicating that NAB1 is might not be regulated via the same pathway as the factors involved in the carbon concentrating mechanism. This conclusion is further discussed in section 6.2.1.

Also light responsive elements can be detected on the *NAB1* promoter; for instance the GATA-box, which is conserved in LHCII genes of vascular plants, starting at position -415 bp, and the GT1 consensus GRWAAW from -529 bp on, which is found in several light responsive genes of vascular plants (Zhou, 1999). These sequences are however not encoded in the 255 bp fragment of the *LHCBM6* promoter, which was shown to be sufficient to drive light-dependent transcription (Hahn and Kück, 1999). It might therefore be questionable whether these elements are conserved in *C. reinhardtii*. More important, empirical data suggest that under low and medium light, NAB1 expression does not alter much (Figure 5-5). Under high light however, NAB1 mRNA and protein levels are low (Figure 4-10), but it is not clear whether promoter activity is altered and/or transcripts are degraded.

To summarize, NAB1 mediated translation repression is crucial for PSII antenna size adjustments under varying carbon supply (4.2), and independent analyses by other authors confirm the increased NAB1 expression under CO_2 limitation (Winck et al., 2013a). A 152 bp sequence upstream of the NAB1 translation start site is sufficient to drive expression of the reporter gene *g*Luc in a carbon dioxide dependent manner (4.3.2). This promoter fragment can now be used as a probe to study the binding of transcription factors that are involved in the increased *NAB1* promoter activity and that are therefore part of the signaling cascade under carbon dioxide limitation (7). Furthermore hypoxia, low temperatures and high light stress could be additional factors influencing *NAB1* promoter activity. The cell lines harboring the reporter constructs created in this thesis can be used in future work to test the responsiveness to these abiotic factors, and signaling studies using specific inhibitors could be applied to elucidate the interorganellar communication pathways involved (7).

5 Light dependent redox control of photon capture capacity

5.1 Significance

Light availability is a main factor determining photosynthetic performances and altering cellular redox states (Anderson et al. 1995). Plants and algae therefore constantly adapt their photosynthetic apparatus to ensure balanced photon capture (1.2). However, understanding the regulatory network behind the adjustment of light-harvesting under fluctuating light is, despite extensive research, still in its infancies (Leister et al 2012; Dietz et al., 2015; 1.3). In this chapter, details of a light dependent redox based control of LHCBM protein synthesis were elucidated, providing insights into retrograde signaling pathways between chloroplast and cytosol. The results presented in section 5.2 were obtained in collaboration as described below.

5.2 Unpublished results II

A light switch based on protein S-nitrosylation fine-tunes photosynthetic light-harvesting in the microalga *Chlamydomonas reinhardtii*

Authors

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Author contributions

The experiments were designed by Hanna Berger, Marcello de Mia, Samuel Morisse, Christophe Marchand, Stephane Lemaire, Lutz Wobbe, and Olaf Kruse.

The experiments were performed by Hanna Berger, Marcello de Mia, Samuel Morisse, and Christophe Marchand. Hanna Berger coordinated the collaboration and performed *in silico* protein analyses (Figure 5-1), purification of recombinant NAB1 protein, as well as protein analyses via gel electrophoresis and immunoblot detection in whole cell lysates to study accumulation of light-harvesting proteins *in vivo* (Figures 5-3B and 5-5). Hanna Berger also depicted the model (Figure 5-7). Samuel Morisse and to the major part Marcello de Mia, provided data on *in vitro* and *in vivo* nitrosylation of NAB1 (Figures 5-2B, 5-3A and 5-4) as well as

thioredoxin mediated denitrosylation (Figure 5-6), using the biotin-switch technique in combination with affinity chromatography and immunoblot detection. Christophe Marchand performed mass spectrometry analyses (Figure 5-2A).

Data were analyzed and interpreted by Hanna Berger, Marcello de Mia, Samuel Morisse, Christophe Marchand, Stephane Lemaire, Lutz Wobbe, and Olaf Kruse.

The manuscript was written by Hanna Berger, Lutz Wobbe, Stephane Lemaire, and Olaf Kruse.

Abstract

Photosynthetic eukaryotes are constantly challenged with a fluctuating supply of light in their natural environment. Key components of the photosynthetic apparatus are encoded by the nuclear instead of the chloroplast genome, adding another layer of complexity to long-term photoacclimation processes which adjust the stoichiometry of the photosynthetic machinery. Here, we provide clear evidence for a regulatory circuit that modulates the cytosolic synthesis of photosystem II associated major light-harvesting proteins (LHCII) in response to light quantity changes. In *Chlamydomonas reinhardtii*, the RNA binding protein NAB1 resides in the cytosol and represses translation of certain *LHCII* isoform mRNAs. Specific nitrosylation of cysteine 226 reduces the repressor activity and could be demonstrated *in vitro* and *in vivo*. Our data indicate that the extent of NAB1 nitrosylation found *in vivo* is fine-tuned in a light-dependent fashion by recruiting cytosolic thioredoxin h1 and NADPH dependent thioredoxin reductase to activate NAB1 via denitrosylation in response to an increased light supply. This redox control of cytosolic protein synthesis in concert with nuclear transcription allows adjusting the amount of LHCII to the cellular energy demand under fluctuating light.

Introduction

Photosynthetic organisms constantly adapt their light harvesting machinery to a changing environment to ensure optimal photosynthetic efficiency (Anderson et al., 1995). Expression control of the major light harvesting proteins at photosystem II (LHCII) provides an efficient means to regulate the initial step of photosynthesis.

In unicellular green algae, including the model organism *Chlamydomonas reinhardtii*, exposure to high light causes a precipitous drop in LHCII transcript levels, while a reduced light availability has an opposite effect (Escoubas et al., 1995; Durnford et al., 2003; Chen et al., 2004). This strong modulation of transcript levels is mainly achieved by controlling nuclear transcription initiation rates (Escoubas et al., 1995), which is in agreement with an unchanged stability of LHCII transcripts in high light grown *C. reinhardtii* cells (Durnford et al., 2003). In this alga, also translation of LHCII encoding mRNAs is controlled by light and its repression occurs after elevating light intensity (Durnford et al., 2003; Mussgnug et al., 2005).

This organism contains the cytosolic RNA binding protein NAB1, which acts as a translation repressor by sequestrating LHCBM mRNAs into translationally silent messenger ribonucleoprotein complexes, thus preventing their translation (Mussgnug et al., 2005). NAB1 shows a preference for certain LHCBM isoform transcripts and in accordance with its function as a LHCII translation repressor, the *NAB1* knock out mutant over-accumulates light-harvesting proteins, displayed by a dark green phenotype (Mussgnug et al., 2005). The extent of NAB1 mediated translation repression is controlled on multiple levels, which perfectly reflects the vital role that light-harvesting plays in a phototrophic organisms (Wobbe et al., 2009; Blifernez et al., 2011; Berger et al., 2014).

For instance carbon dioxide limiting conditions trigger the accumulation of NAB1 via nuclear promoter activation, which in turn reduces the synthesis of LHCII proteins when antenna size reduction is required to maintain a normal PSII excitation pressure, while the reductant sink capacity of the Calvin cycle is restricted (Berger et al., 2014). Promoter activation can be abolished by blocking photosynthetic electron transport, which indicates that NAB1 expression is modulated by chloroplast retrograde signals and several lines of evidence suggest that NAB1 can be regarded as a regulatory hub connecting short- and long-term photosynthetic acclimation mechanisms.

RNA binding activity of NAB1 is regulated by two types of post-translational modification with arginine methylation acting as a master switch that adjusts activity to the prevailing metabolic situation (Blifernez et al., 2011). In addition, NAB1 activity is redox controlled via modification two cysteines at amino acid position 181 and 226, located in the RNA recognition motif (RRM)

(Wobbe et al., 2009). Oxidative modification of these cysteines reduces the RNA binding and translation repressor activity of NAB1. Replacement of either amino acid with serine impairs NAB1 deactivation, causing a pale green, small antenna phenotype (Beckmann et al., 2009; Wobbe et al., 2009), but single mutation of C226 causes a stronger phenotype than the respective exchange of C181 (Wobbe et al., 2009). Together with biochemical data, the phenotypic difference between both cysteine single mutants demonstrated that single cysteine modification and not the formation of intramolecular disulfide bonds must be the mechanisms of NAB1 deactivation (Wobbe et al., 2009). Distinct types of single cysteine modification are known and glutathionylation as well as nitrosylation represent prominent examples. Protein glutathionylation consists of the formation of mixed disulfides between glutathione (GSH; c-L-glutamyl-L-cysteinylglycine) and a protein thiol. This modification can protect protein thiols from over-oxidation, but can also have a regulative function (Zaffagnini et al., 2012). Protein S-nitrosylation, results from the covalent binding of nitric oxide (NO) to a cysteine thiol moiety and plays a major role in numerous cellular processes in mammals (Benhar et al., 2009). NO production has also been reported in plants where it has been detected in different tissues or subcellular compartments, such as plastids, peroxisomes or mitochondria (Baudouin, 2011; Fröhlich and Durner, 2011). In animals, the main source of NO is a reaction catalyzed by NO synthases (NOS), but the relevance of this synthesis pathway for plant NO metabolism is still a matter of debate (Fröhlich and Durner, 2011). Although a NOS homologue could be characterized in the microalga *Ostreococcus tauri* (Foresi et al., 2010), nitrite and nitrate reductase seem to be the main NO-producing systems in the plant kingdom (Baudouin, 2011; Fröhlich and Durner, 2011), where a multitude of physiological processes have been shown to rely on NO signaling (Astier et al., 2011). Nitric oxide cannot only react with protein thiols directly to form protein S-nitrosothiols, but can also form adducts with the most abundant intracellular cysteine-containing tripeptide glutathione. S-nitrosoglutathione (GSNO) is regarded as the main mobile NO reservoir of the cell and a major trans-nitrosylating agent. Its concentration is controlled by GSNO reductase (GSNOR), which catalyzes the reduction of GSNO to oxidized glutathione (GSSG) and ammonia, thereby modulating indirectly the level of nitrosylated proteins (Liu et al., 2001). Recently, a proteomic study conducted with *C. reinhardtii* demonstrated that S-nitrosylation could constitute a major regulatory mechanism, when this organism is exposed to nitrosative stress and large number of identified S-nitrosylation targets are implicated in photosynthesis (Morisse et al., 2014). In the present study we set out to determine the physiological role and type of single cysteine modification occurring on NAB1 *in vivo*. Overall our data clearly demonstrate that reversible nitrosylation of a specific cysteine residue in the RRM domain of NAB1 modulates translation of light-harvesting protein-encoding mRNAs in the cytosol of *C. reinhardtii* cells. The

fact that denitrosylation of NAB1, which results in its activation, requires a cytosolic thioredoxin system provides important insights into the cross-talk between chloroplast and cytosol, which is needed for a stoichiometric fine-tuning of photosynthetic apparatus components, encoded by the nuclear genome.

Methods

Chemicals and enzymes

Reduced glutathione (GSH), dithiothreitol (DTT), ascorbate (ASC), nicotinamide adenine dinucleotide phosphate (NADPH), 1,1-Diethyl-2-hydroxy-2-nitroso-hydrazine sodium salt (DEA-NONOate), 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), and nitrosogluthatione (GSNO) were purchased from Sigma. GSNO and NADPH concentrations were determined spectrophotometrically using molar extinction coefficients of 920 M^{-1} cm^{-1} at 335 nm for the former and 6230 M^{-1} cm^{-1} at 340 nm for the latter. N-[6-(Biotinamido)hexyl]-3'-(2'-pyridyldithio)proprionamide (HPDP-biotin) was purchased from Pierce. Recombinant proteins were prepared as previously described for TRX h1 from *C. reinhardtii* (Goyer et al., 1999), NADPH-dependent thioredoxin reductase (NTR) from *Saccharomyces cerevisiae* (Pérez-Pérez et al., 2014) and cytoplasmic glyceraldehyde-3-phosphate dehydrogenase (GAPC1) from *Arabidopsis thaliana* (Bedhomme et al., 2012).

Strains and Culture Conditions

The *Chlamydomonas reinhardtii* strain CC849 (cw10; mt-; Chlamydomonas resource center, St. Paul, MN, USA) served as wild-type and is the parental strain of the *NAB1* knock out mutant *stm3* (Mussgnug et al., 2005). The cysteine mutants, carrying NAB1 with either C181 or C226 replaced by serine, as well as the respective control strain with a wild-type NAB1, were created by introducing the *NAB1* gene under the control of *PSAD* promotor into *stm3* (Wobbe et al., 2009), thereby obviating endogenous expression regulation. To distinguish between LHC transcription and translation control, HA-tagged LHCBM6 under the control of *PSAD* promotor was introduced into the wild-type strain, *NAB1* k.o. and the cysteine mutants (Mussgnug et al., 2005; Wobbe et al., 2009).

The strains were cultured in tris-acetate-phosphate (TAP) media under low (40 µmol photons m^{-2} s^{-1}) or elevated (200 µmol phototns m^{-2} s^{-1}) fluorescent white light. Cell density was monitored by determining the optical density at 750 nm as well as cell count (Beckmann coulter). For artificial nitrosative stress, 2 mM GSNO or 1 mM DEA-NONOate was added to the cultures. Concentration was checked photometrically using the extinction coefficients at 355 and 250 nm, respectively. To prevent nitrosylation, 1 mM cPTIO was added as NO scavenger.

In vitro glutathionylation of NAB1 and GAPC1

Reduced recombinant proteins (Mussgnug et al., 2005; Bedhomme et al., 2012; 40 µM) were incubated in 30 mM Tris-HCl (pH 7.9), 1 mM EDTA in the presence of 0.1 mM H_2O_2 and 0.5 mM GSH for 30 min at 25 °C. For each protein a control was performed by replacing H_2O_2 and GSH with Tris-HCl buffer and the reversibility of the induced glutathionylation was verified after incubation with 2.5 mM dithiotreitol for 15 min at 25 °C. After each treatment proteins were subjected to MALDI-TOF MS analysis as described below.

MALDI-TOF mass spectrometry

1 µL of protein solution was mixed with 4 µL of a saturated solution of sinapinic acid in 30% acetonitrile containing 0.3% trifluoroacetic acid. Then, 1.5 µL of this premix was deposited onto the sample plate and allowed to dry under a gentle air stream at room temperature. Mass determination of NAB1 and GAPC1 was carried out after calibration on mono-charged and di-charged yeast enolase ions (m/z: 23336 and 46671 Da, respectively) in positive linear mode on a Performance Axima MALDI-TOF mass spectrometer (Shimadzu, Manchester, United Kingdom) with a pulse-extraction fixed at 28000.

Nitrosylation/Denitrosylation treatments

Purified recombinant NAB1 (Mussgnug et al., 2005) and GAPC1 (Bedhomme et al., 2012) were nitrosylated in sample buffer (30 mM Tris-HCl pH 7.5, EDTA 1 mM, NaCl 100 mM) in the presence of 2 mM GSNO for 30 minutes in the dark at 25 °C. Denitrosylation of NAB1-SNO and GAPC1-SNO were performed for 30 minutes in sample buffer supplemented with GSH (5 mM) or DTT (20 mM) or with a mixture containing 1 µM NTR and 2 mM NADPH in the presence or absence of 20 µM TRX h1.

Biotin switch assay

The extent of protein nitrosylation was assessed by the adapted biotin switch assay (Jaffrey and Snyder, 2001). After nitrosylation/denitrosylation treatments, proteins (0.8-1 mg/mL) were precipitated with 4 volumes of cold acetone at -20°C during 20 min and pelleted by centrifugation at 4 °C for 10 min at 15,000 x*g*. The pellet was resuspended in TENS buffer (30 mM Tris-HCl pH 7.9, 1 mM EDTA, 100 mM NaCl and 1% SDS) supplemented with a cocktail of alkylating reagents (10 mM iodoacetamide, 10 mM N-ethylmaleimide), to allow blocking of free thiols. After 30 minutes incubation at 25 °C under shaking, the samples were acetone precipitated, as described above, to remove unreacted alkylating reagents. After resuspension in TENS buffer, proteins were incubated in the presence of 40 mM ascorbate and 1 mM HPDP-biotin for 30 min. This step allows reduction of S-nitrosylated cysteines and their

derivatization with biotin. Proteins were then acetone precipitated to remove unreacted labeling compounds, pelleted by centrifugation as above and resuspended in TENS buffer. All steps were performed in the dark. After the final precipitation, proteins were analyzed by gel electrophoresis and immunoblotting.

All BST assays included a negative control where ascorbate was omitted to prevent reduction of S-nitrosothiols and subsequent biotinylation. This control without ascorbate allows assessing the efficiency of the initial thiol-blocking step.

Gel electrophoresis and Immunoblotting

Whole cell lysates were subjected to immunoblotting as described (Wobbe et al., 2009; Blifernez et al., 2011). Tris-glycine polyacryamid gels (Laemmli, 1970) were used for coomassie stains and blots against NAB1 and LHCBM6/8, tris-tricine polyacryamid gels (Schägger and von Jagow, 1987) were used for detection of HA-tagged LHCBM6. Antiserum raised against NAB1 was generated as described (Mussgnug et al., 2005) and anti-LHCBM6/8 antibody was a gift of M. Hippler (Münster, Germany). The antibody detecting the HA-tag was purchased (Roche).

For nitrosylation/denitrosylation assays, proteins were quantified using the bicinchoninic acid assay, separated by non-reducing SDS-PAGE and transferred onto nitrocellulose membranes. Protein loading and transfer were assessed by Ponceau staining of the membrane. Proteins were then analyzed by western blotting using a primary anti-biotin antibody (1:5,000 dilution; Sigma-Aldrich) and an anti-mouse secondary antibody coupled to peroxidase (1:10,000 dilution; Sigma-Aldrich). Signals were visualized by enhanced chemiluminescence, as described previously (Bedhomme et al., 2012).

Coomassie brilliant blue staining of gels or Ponceau staining of membranes served as loading control.

In silico protein analysis

ExPASy tools were used for protein *in silico* analysis. The predicted structure of the NAB1 RRM domain was visualized with Swiss pdb viewer (Guex and Peitsch, 1997) available at http://www.expasy.org/spdbv/ . Grantham polarity plot was obtained from http://web.expasy.org/protscale/ with a window size of nine amino acids (Gasteiger et al., 2005).

Results

Cysteine 226 is located in a protein microenvironment frequently found surrounding S-nitrosylation sites

In a previous study, we showed that the cytosolic protein NAB1 has a central role as a control hub in the complex regulatory network responsible for adjusting the light-harvesting antenna system to changing environmental conditions. In particular the redox state of two cysteines at amino acid positions 181 and 226 was shown to be critical for the LHCII translation repressor activity of NAB1 (Wobbe et al., 2009). Expression of three distinct NAB1 cysteine mutants C181S, C226S and C181/226S in a mutant lacking endogenous NAB1 revealed a pale green phenotype in case of the double mutant and single mutant C226S (Figure 5-1A). The serine residues replacing cysteines in these NAB1 variants mimic the free SH-state, which was shown to be the more active state in RNA binding studies (Wobbe et al., 2009). In contrast to their SH-containing counterparts, the serines are not amenable to oxidative modifications typically seen for cysteines. The low chlorophyll content displayed by mutants C226S and C181/226S is the result of a reduced light-harvesting protein (LHCBM) accumulation which can in turn be explained by the disruption of a crucial deactivation mechanism and translation repression being locked in the 'on' state (Wobbe et al., 2009). The phenotype of single cysteine mutant C226S clearly shows that this residue alone is sufficient for a redox control of NAB1 activity.

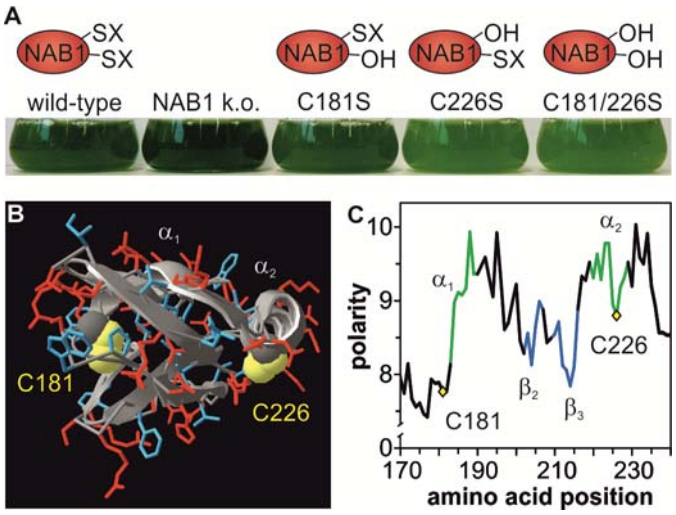

Figure 5-1 Essentiality of C226 for NAB1 redox control and *in silico* indication for its nitrosylation.
(A) Pale green phenotype displayed by cell lines expressing NAB1 variants that lack C226, which is essential for cysteine based redox control of its repressor activity. In these variants, cysteines amenable to redox based modifications (-SX) are replaced with non-reative serines (-OH) that mimic the free thiol state (-SH). (B) *In silico* model of the NAB1-RRM domain created with swiss pdb-viewer. Polar and charged amino acids in the surrounding of cysteines 181 and 226 are shown in red and uncharged/non-polar amino acids are depicted in blue. The protein backbone is shown in a ribbon presentation. (C) Polarity plot of the RRM domain. The relative polarity is shown on the y-axis, while the amino acid position is given on the x-axis. Alpha-helices, β-sheets and loop regions are indicated by a green, blue and black color, respectively.

Previous *in silico* analyses based on homology modelling of the C-terminal RRM domain of NAB1, which harbors both cysteines, demonstrated a distinct surface exposure of C181 and C226. In line with its more crucial function for NAB1 redox control, C226 turned out to be the more accessible cysteine (Wobbe et al., 2009). Apart from the higher reactivity predicted for C226, the great spatial distance between C181 and C226 indicated that intramolecular disulfide formation cannot occur and this view was further supported by *in vitro* experiments conducted with recombinant NAB1 (Wobbe et al., 2009). Glutathionylation of NAB1 could be demonstrated after treatment of recombinant NAB1 with glutathione disulfide, but if this type of modification is relevant for NAB1 redox control *in vivo* remained unclear (Wobbe et al., 2009). As a starting point for further investigations regarding the precise chemical nature of C226 modifications controlling NAB1 activity *in vivo*, we extended previous *in silico* analyses by inspecting the microenvironment of both cysteines more closely (Figure 5-1B). C181 is surrounded by non-polar amino acids (Figure 5-1C; blue residues in Figure 5-1B) in an environment with a low electrostatic potential, whereas the environment of C226 is characterized by a high electrostatic potential (Figure 5-1C). Out of fourteen neighboring residues (-7 to +7), seven are charged and two are polar (red residues Figure 5-1B) in the case of C226. This cysteine is part of an α-helix (α_2, Figure 5-1B) in a rather surface exposed area, overall an environment with characteristics of S-nitrosylation sites (Gould et al., 2013). Futhermore, lysines are found overrepresented in proximity to cysteines amenable to nitrosylation (Lindermayr et al., 2006; Morisse et al., 2014), and relative to C226 in NAB1, two lysines are located at positions -7 (K219) and +2 (K228), respectively. These position-specific characteristics support that C226 rather than C181 is the main target for thiol-based NAB1 activity control, as assumed before (Wobbe et al., 2009), and in addition that C226 is potentially amenable to nitrosylation.

NAB1 is deactivated under nitrosative stress conditions by nitrosylation of C226

Earlier results indicated that NAB1 could be glutathionylated *in vitro* (Wobbe et al., 2009). However, we wanted to reconfirm that C226 of NAB1 can indeed be glutathionylated, since recent *in silico* analyses (Figure 5-1B,C) pointed at nitrosylation as a candidate cysteine modification. For *in vitro* studies, recombinant NAB1 carrying the wild-type sequence was purified (Mussgnug et al., 2005) and treated with glutathione together with hydrogen peroxide (Figure 5-2A, GSH+H_2O_2), allowing efficient glutathionylation of amenable cysteine residues. The recombinant *A. thaliana* protein GAPC1 was used as control since this enzyme undergoes glutathionylation (Bedhomme et al., 2012) in addition to nitrosylation (Zaffagnini et al., 2013). Mass spectrometric showed that GAPC1 is shifted by 305 Dalton after glutathionylation treatment, while this is not the case for NAB1 (Figure 5-2A). This shift is consistent with the

presence of one glutathione adduct per GAPC1 monomer. The missing shift of NAB1 after glutathionylation treatment indicates that this protein is probably amenable to glutathionylation, in contrast to previous results (Wobbe et al., 2009).

To test whether cysteine 226 can be nitrosylated *in vitro*, we applied the NO donor DEA-NONOate in combination with a comparative biotin switch assay (Figure 5-2B). Specific detection of nitrosylated cysteines by the biotin switch technique is based on the specificity of the reaction between ascorbate and nitrosylated thiols (S-NO). After nitrosylation of protein cysteines all remaining free thiols (-SH) are blocked with alkylating agents, before S-NOs are converted back into SH-groups in a transnitrosation reaction with ascorbate. Concomitantly, nascent free thiol groups are quickly tagged with biotin via intermolecular disulfide formation (-S-S-biotin) (Jaffrey and Snyder, 2001). The tag can then be further used to pull-down biotinylated proteins with streptavidin resins.

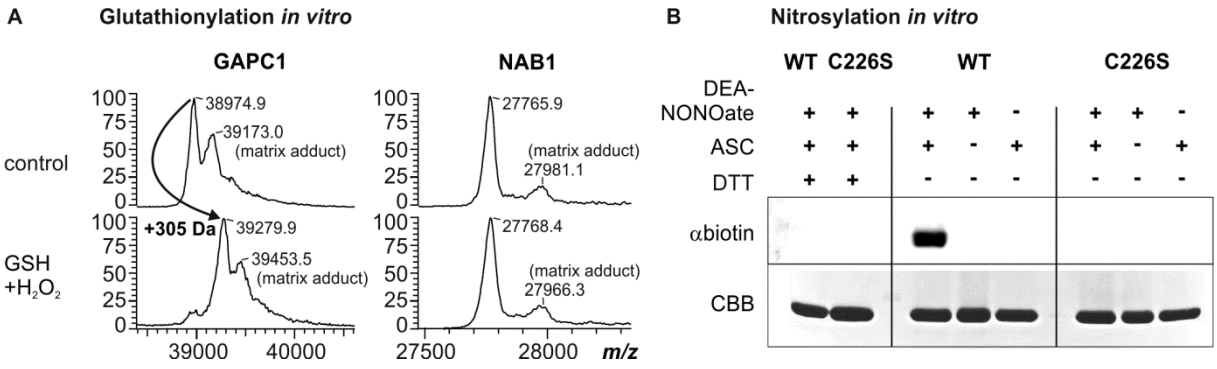

Figure 5-2 NAB1 can be nitrosylated *in vitro*.
(A) Analysis of NAB1 and GAPC1 *in vitro* glutathionylation following treatment with hydrogen peroxide and glutathione via MALDI-TOF mass spectrometry. Left panel: Mass spectrum of GAPC1 obtained after treatment with GSH+H_2O_2 and subsequent re-reduction using dithiotreitol (control). A mass increase of 305 Da corresponds to one glutathione molecule covalently bound per molecule of protein. The peaks labelled 'matrix adducts' correspond to proteins with a sinapinic acid adduct. Differences between mass peaks of unmodified NAB1 and GAPC1 are within the experimental error of the instrument. Right panel: Mass spectrometric analysis of recombinant NAB1 under identical conditions. (B) Treatment of recombinant NAB1 (WT) and $NAB1_{C226S}$ (C226S) with the NO-donor DEA-NONOate followed by the biotin switch assay. Addition (+) or omission (-) of the reaction components DEA-NONOate, ascorbate (Asc) and dithiothreitol (DTT) during the assay is indicated in the upper part. NAB1-biotinylation as an indicator for prior nitrosylation was detected by immunoblotting with a biotin-specific antiserum (middle panel; αbiotin) and NAB1 protein amounts were assessed by coomassie staining after SDS-PAGE separation.

Application of the biotin switch technique subsequent to nitrosylation of recombinant NAB1 with DEA-NONOate allowed biotinylation of Wt-NAB1 (Figure 5-2B, middle panel, WT, +DEA-NONOate, +Asc, αNAB1). As previous results (Wobbe et al., 2009) and *in silico* analyses (Figure 5-1B,C) suggest that the cysteine residue 226 is the main target for redox based NAB1 activity regulation, a recombinant protein variant, in which C226 is replaced by a non-reactive serine (C226S) (Wobbe et al., 2009), was investigated as well. Intriguingly, the $NAB1_{C226S}$ variant was not biotinylation after the identical nitrosylation and biotin switch treatment as wild-type

protein (Figure 5-2B, right panel, C226S). Omission of either NO-donor or ascorbate during the biotin switch assay prevented tagging of NAB1 (Figure 5-2B, WT, +DEA-NONOate/-Asc and -DEA-NONOate/+Asc), demonstrating specificity of the assay. As another control disulfide reduction with DTT was performed to show reversibility of labeling (Figure 5-2B, left panel, WT, C226, +DTT).

To further confirm that cysteine 226 is amenable to nitrosylation, we applied the biotin switch assay to recombinant Wt-NAB1 and $NAB1_{C226S}$ following treatment with S-nitrosoglutathione (GSNO) (Figure 5-3A). Only in the case of Wt-NAB1, exposure to GSNO resulted in a detectable nitrosylation (Figure 5-3A, Wt, C226S, +GSNO). In summary the data demonstrate that cysteine 226 of NAB1 can be nitrosylated *in vitro*.

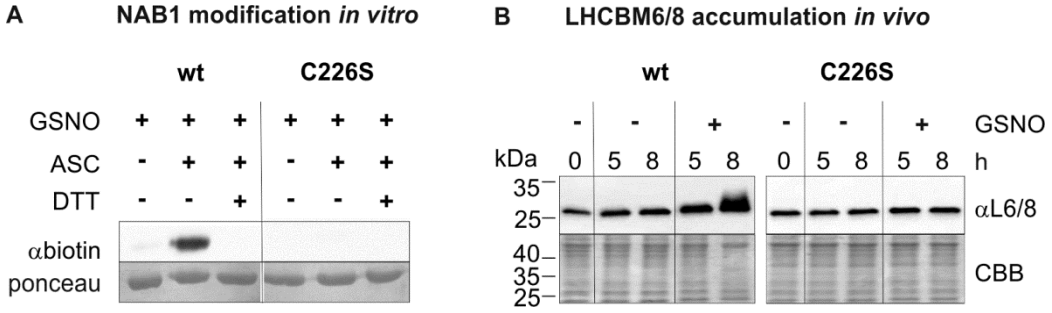

Figure 5-3 Nitrosylation of NAB1 at C226 reduces its translation repressor activity.
(A) In vitro nitrosylation of recombinant NAB1 with S-nitrosoglutathione (GSNO). The wild-type version of NAB1 was used along with $NAB1_{C226S}$. The biotin switch assay was applied to detect S-nitrosylation via immunodetection of the biotin label (αbiotin). Omission of ascorbate (-ASC) and addition of dithiothreitol (+DTT) served as a control to assess stringency of the assay. (B) Effects of C226 nitrosylation on the accumulation of major light harvesting proteins LHCBM6/8. The cellular amount of LHCBM6/8 was determined by immunodetection (αL6/8) 5 and 8 h following GSNO addition (+) to cultures expressing either Wt-NAB1 (wt) or $NAB1_{C226S}$ (C226S) under control of *PSAD* promoter. Negative controls (-GSNO) were included to exclude effects unrelated to nitrosative stress.

We further investigated whether nitrosylation of NAB1 *in vivo* has an impact on its translation repressor activity. Changes in the accumulation of the PSII-associated major light harvesting protein LHCBM6, whose mRNA represents the prime target of NAB1 (Mussgnug et al., 2005) were chosen as a proxy for alterations in NAB1 activity induced by nitrosylation (Figure 5-3B). S-nitrosoglutathione (GSNO) was added to liquid cultures of cells either expressing Wt-NAB1 or the $NAB1_{C226S}$ variant to induce nitrosylation of C226 in Wt-NAB1. In the time range of five to eight hours, a strong accumulation of LHCBM6/8 was detected in cultures expressing Wt-NAB1 after adding GSNO (Figure 5-3B, wt, +GSNO, 5 and 8 h), whereas GSNO addition had no effect on LHCBM6/8 accumulation in cultures of $NAB1_{C226S}$ (Figure 5-3B, C226S). This result indicates that artificial *in vivo* nitrosylation of NAB1 at cysteine 226 leads to a decreased repressor activity followed by an uncontrolled accumulation of light harvesting proteins.

Light intensity modulates the degree of NAB1 nitrosylation

The results presented in Figures 5-2 and 5-3 demonstrate that NAB1 can be nitrosylated artificially *in vitro* using nitrosylating agents and that nitrosative stress triggers the deactivation of NAB1 *in vivo*. To investigate if NAB1 is nitrosylated *in vivo* under physiological conditions, the degree of nitrosylation was analyzed under different light conditions using the biotin switch assay (Figure 5-4). Cultivation of wild-type cells under elevated light conditions (200 µmol m^{-2} s^{-1}) was not accompanied by a detectable nitrosylation of NAB1 (Figure 5-4, wt, EL, Out). A reduction of the light intensity to 40 µmol m^{-2} s^{-1} during cultivation, however, caused nitrosylation of Wt-NAB1 (Figure 5-4, wt, LL, Out). Absence of nitrosylation in $NAB1_{C226S}$ under these conditions again underscored the relevance of C226 for NAB1 redox control by cysteine nitrosylation (Figure 5-4, C226, LL, Out).

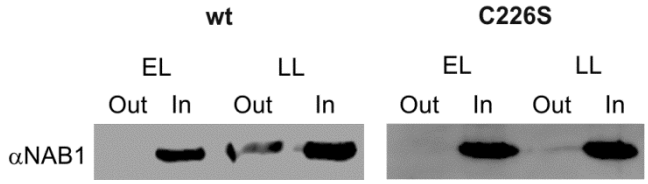

Figure 5-4 NAB1 is nitrosylated *in vivo* at C226 under low light conditions.
Chlamydomonas cells expressing Wt-NAB1 or $NAB1_{C226S}$ from the *PSAD* promoter were grown under low light (LL, 40 µmol m^{-2} sec^{-1}) or elevated light (EL, 200 µmol m^{-2} sec^{-1}) for eight hours and then subjected to the biotin switch assay. After biotin switch, biotinylated proteins were purified by streptavidin affinity chromatography and eluted with DTT. Eluted proteins were analyzed by western blot using anti-NAB1 antibodies (αNAB1). In: Input of the column; Out: Eluate of the column.

The finding that NAB1 is only nitrosylated under low light conditions provided novel insights into the physiology behind nitrosylation-dependent redox control of NAB1. From a physiological point of view, a reduced activity of NAB1 under low light conditions enables the assembly of a large antenna needed for efficient light harvesting when light availability is low. An absence of nitrosylation under elevated light conditions permits translation control of light harvesting proteins, which is required for efficient antenna size reduction as part of the acclimation mechanisms that adjust the photosynthetic apparatus to the increased availability of light. Reversible nitrosylation could therefore be a fast and efficient means to either permit translation or sequester preformed LHCBM mRNAs in untranslated mRNP complexes (Mussgnug et al., 2005).

Low light-induced deactivation of NAB1 requires the intracellular production of nitric oxide

The LHCBM6 translation repressor NAB1 is clearly nitrosylated under low light (Figure 5-4), primarily at C226 (Figures 5-1 to 5-4), and as an oxidative modification this lowers its RNA binding activity (Wobbe et al., 2009) (Figure 5-3B). It is well known that *C. reinhardtii* cells accumulate LHCBM proteins in response to light limitation and a higher transcription rate of

LHCBM genes contributes to the observed accumulation (Escoubas et al., 1995; Teramoto et al., 2002). We next wanted to answer the question, if a low light-induced deactivation of translation control via NAB1 nitrosylation is needed for antenna size enlargement in addition to *LHCBM* promoter activation. To this end, algal strains devoid of NAB1 (*NAB1* k.o.), carrying the wild-type (wt) protein or mutated versions (C181S and C226S) were subjected to a shift from elevated to low light (Figure 5-5) Into all strains a HA-tagged LHCBM6 version expressed under control of the constitutive *PSAD* promotor was introduced to allow differentiation between LHCII transcription and translation control. Algal cultures were grown under elevated light conditions (200 µmol m^{-2} s^{-1}) before transferring them to low light intensities (40 µmol m^{-2} s^{-1}) or continuing elevated light cultivation for five hours. The nitric oxide scavenger cPTIO was applied to inhibit nitrosylation of NAB1 (Figure 5-5A).

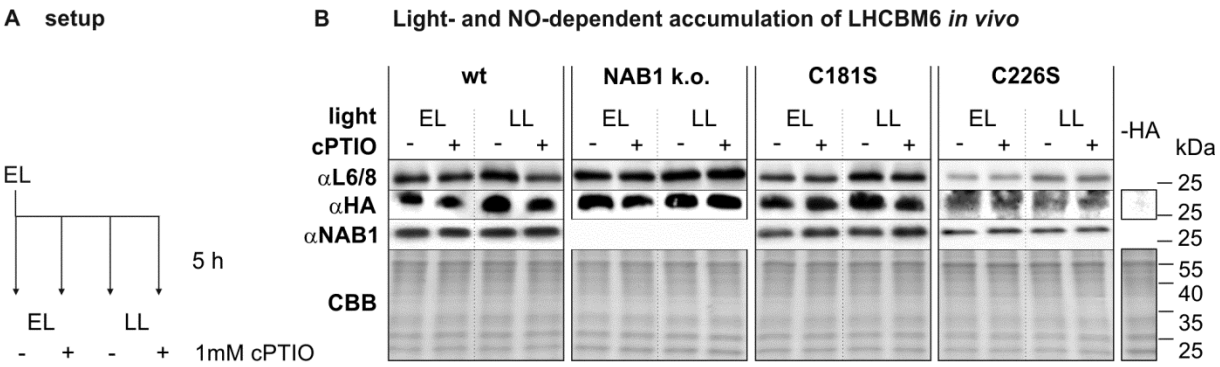

Figure 5-5 LHCBM6 accumulation under low light is partially triggered by NAB1 nitrosylation.
Algal strains without NAB1 (NAB1 k.o.), expressing the wild-type (wt) protein or mutated versions (C181S and C226S) were used. A wild-type strain expressing NAB1 from the endogenous promoter was used here to determine light-dependent NAB1 expression. All strains additionally contain a HA-tagged LHCBM6 under control of the constitutive *PSAD* promotor. (A) Experimental setup: Strains were precultivated under elevated light (EL, 200 µmol m^{-2} s^{-1}) and either remained in EL or were shifted to low light intensities (LL, 40 µmol m^{-2} s^{-1}) for 5 h, with (+) or without (-) the addition of 1mM cPTIO. (B) Immunoblot analysis of LHCBM6/8 (αL6/8), LHCBM6-HA (αHA), NAB1 (αNAB1) and a Coomassie brilliant blue (CBB) stain serving as a loading control. To probe specificity of HA-tag immunodetection, a strain without LHCBM6-HA was loaded as negative control (-HA).

When cultures expressing Wt-NAB1 were subjected to the shift from elevated to low light, LHCBM6/8 levels rose as expected (Figure 5-5B, wt, αL6/8, EL vs. LL, -cPTIO). A strong accumulation of the HA-tagged LHCBM6 protein (αHA) under these conditions, whose expression is decoupled from endogenous *LHCBM* promoter control, indicated that a de-repression of translation could significantly contribute to the observed LHCBM6 accumulation. As was recently shown, cellular NAB1 levels determine the extent of LHCBM translation repression under certain conditions (Berger et al., 2014). The shift to low light, however, did not result in a changed NAB1 level (αNAB1) and indicated that an altered repressor activity is the main trigger for LHCBM6 accumulation. This could be confirmed by supplementing cultures with the NO scavenger cPTIO to inhibit NAB1 nitrosylation during low light acclimation, which completely prevented accumulation of LHCBM6 and HA-LHCBM6 (Figure 5-5B, LL, +cPTIO,

αL6/8, αHA). A similar effect of cPTIO was noted for the strain expressing a $NAB1_{C181S}$ variant (C181S, LL, + vs. –cPTIO), but could not be observed in the case of $NAB1_{C226S}$ (C226S, + vs. -cPTIO). Further supporting the NO-dependent specific nitrosylation of NAB1 at cysteine 226, nitric oxide scavenging had no effect on LHCBM6 accumulation in the NAB1 knock out mutant (*NAB1* k.o., + vs. – cPTIO).

We therefore conclude that under the condition tested, the RNA binding activity of NAB1 is reduced by modification of cysteine 226 and that this modification is indeed based on nitrosylation.

*NAB1 can be efficiently denitrosylated by thiore*do*xin h1 in vitro*

Results presented in Figure 5-5 clearly indicate that NAB1 undergoes nitrosylation, when *Chlamydomonas* cultures are transferred from higher to lower light intensities. The resulting reduction in NAB1 repressor activity ensures that translation repression of light-harvesting protein encoding mRNAs is relieved and that LHCBM proteins accumulate in *C. reinhardtii* cells exposed to limiting light supply. We next wanted to address the molecular mechanisms behind the reverse process that re-activates NAB1 by denitrosylation in response to an increased supply of light (Figure 5-4). The best described denitrosylation mechanisms occurring in eukaryotic cells operate via glutathione (GSH) in conjunction with the enzyme S-nitrosglutathione reductase (GSNOR) or the thioredoxin/thioredoxin reductase system (Benhar et al., 2009).

To gain insight into the mechanisms activating NAB1 in cells exposed to elevated light quantities, we analyzed if glutathione or a thioredoxin reducing system are capable of denitrosylating NAB1 *in vitro* (Figure 5-6). Recombinant NAB1 was nitrosylated at cysteine 226 by adding S-nitrosoglutathione (Figure 5-6, +GSNO) and directly subjected to the biotin switch assay (Figure 5-6, lanes 1-3) or treated with either glutathione (lane 4, +GSH) or cytosolic thioredoxin h1 in combination with a thioredoxin re-reduction system (lane 5, +TRX h1, + NTR-NADPH) prior to the detection of protein nitrosylation.

Nitrosylated NAB1 could be efficiently denitrosylated only when thioredoxin h1 was provided together with a NADPH-dependent thioredoxin reductase (NTR) and NADPH (Figure 5-6, lane 5). In contrast, addition of glutathione had no significant effect on NAB1 nitrosylation, whereas nitrosylation of a cytosolic glyceraldehyde-3-phosphate dehydrogenase from *A. thaliana* (Figure 5-6, GAPC1) was reduced to undetectable levels only following glutathione treatment (lane 4), but not when the thioredoxin system was used (lane 5). The specific denitrosylation of GAPC1 by glutathione has been demonstrated before (Zaffagnini et al., 2013), and confirms reliability of the *in vitro* assay. As additional specificity controls for the assay, the absence of biotin-label on NAB1 or GAPC1 cysteines was demonstrated after omitting ascorbate

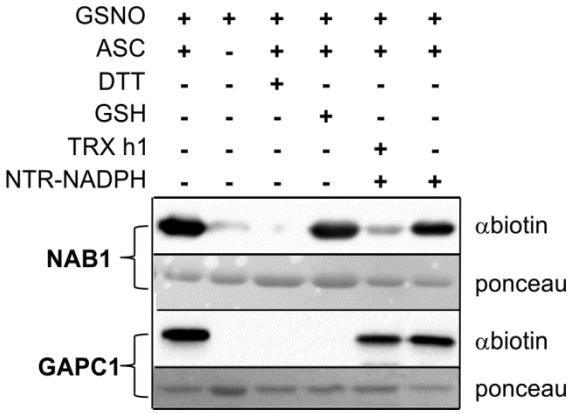

Figure 5-6 Thioredoxin h1 denitrosylates NAB1 *in vitro*.
The purified recombinant proteins NAB1 and GAPC1 were nitrosylated with S-nitrosoglutathione *in vitro* (+GSNO, lanes 1 to 6) and subjected to the biotin switch assay (+GSNO,+ASC) for tagging of S-nitrosylation sites with biotin prior to immunodetection (αbiotin). Specificity of the assay was confirmed by omitting ascorbate (-ASC, lane 2) or including dithiothreitol (+DTT, lane 3) and protein amounts on the blotting membrane were visualized by Ponceau staining. Reduced glutathione (GSH), recombinant thioredoxin h1 (TRX h1) alone or in combination with a complete thioredoxin reducing system (NTR-NADPH) were tested for their denitrosylation capacity with NAB1-SNO and GAPC1-SNO prior to application of the biotin switch assay (lanes 4 to 6).

(Figure 5-6, lane 2, -ASC) or adding dithiothreitol (lane 3, -DTT). NAB1 denitrosylation could only be observed when both thioredoxin h1 and a re-reduction system were present (Figure 5-6, lane 5 vs. lane 6). The finding that NAB1 is efficiently denitrosylated by the cytosolic thioredoxin h1/NADPH:thioredoxin reductase system *in vitro* provides new insights into the chloroplast-to-cytosol cross-talk which fine-tunes cytosolic translation of nucleus encoded light-harvesting proteins.

Discussion

In photosynthetic organisms, long-term acclimation to changes in the availability of light or other external factors having a strong impact on photosynthetic activity, requires a modulated expression of genes encoding components of the photosynthetic apparatus (Woodson and Chory, 2008). The resulting stoichiometric adjustments ensure sufficient photosynthetic performance under adverse conditions and help preventing damage to the photosynthetic machinery induced by the formation of reactive oxygen species (Anderson et al., 1995). During evolution many endosymbiont genes encoding essential photosynthetic proteins have been transferred to the nucleus. A tight regulation of their expression in response to environmental changes requires a cross-talk between different cellular compartments including nucleus, chloroplast, cytosol and also mitochondria (Woodson and Chory, 2008). The amount of PSII associated major light-harvesting proteins determines the light capture capacity of algal and leaf cells and the adjustment of cellular LHCBM levels represents a prime example for the molecular complexity of long-term acclimation processes (Erickson et al., 2015). In *C. reinhardtii*, NAB1 was determined to be a central hub within the network controlling light-harvesting protein

availability in the thylakoid membrane (Mussgnug et al., 2005; Wobbe et al., 2009; Blifernez et al., 2011; Berger et al., 2014). In accordance with its vital function, NAB1 mediated translation control of LHCBM proteins was shown to be itself regulated on multiple levels (Wobbe et al., 2009; Blifernez et al., 2011; Berger et al., 2014). Apart from a modulation of cellular NAB1 levels during the acclimation to an altered carbon availability (Berger et al., 2014), the activity state of NAB1, regulated via post-translational modifications, was demonstrated to determine the translational status of *LHCBM* mRNAs (Wobbe et al., 2009; Blifernez et al., 2011). Two types of post-translational modifications (PTMs) with distinct physiological functions were identified. Arginine methylation is a costly PTM, as measured by the ATP input required, and has a rather low turnover rate (Fackelmayer, 2005). Methylation and demethylation of NAB1 arginine residues can therefore be considered a less dynamic process, which supports the view that this type of modification acts as a master control switch (Blifernez et al., 2011). Indeed, the degree of NAB1 methylation was found to be low when *C. reinhardtii* cells were cultivated under strictly heterotrophic conditions, whereas photoautotrophic growth was accompanied by a high extent of arginine methylation (Blifernez et al., 2011). High methylation levels under photoautotrophic conditions ensure that the slowly responding activity switch is in the 'on' state, when a fine-tuning of light-harvesting plays a vital role for the algal cell.

Unperturbed photoautotrophic growth in a natural environment, however, requires a continuous and fast re-adjustment of LHCBM translation rates in the cytosol to perfectly meet the current demand for light-harvesting proteins in the chloroplast, which is defined by factors such as light availability. Redox control via cysteine modification is a typical example for a dynamic type of protein activity control (Spadaro et al., 2010). In addition to arginine methylation, a second activity control switch has been identified in NAB1 and is based on the reversible modification of cysteine residues present in its RRM domain, as was shown in a previous study (Wobbe et al., 2009). In this study, the general significance of cysteine modification for NAB1 activity control and the more critical role of cysteine 226 could be revealed, but the precise chemical nature of cysteine modifications occurring on NAB1 *in vivo* and their function in a physiological context remained to be elucidated (Wobbe et al., 2009). Inspection of a structural model of the NAB1-RRM domain generated *in silico* together with an analysis of the protein microenvironment surrounding Cys226 suggested that S-nitrosylation could preferentially occur at this site (Figure 5-1). This was confirmed by demonstrating specific nitrosylation of cysteine 226 *in vitro* using nitrosylating agents (Figures 5-2B and 5-3A). Nitrosative stress resulted in a strong accumulation of LHCBM6/8 proteins, which is the prime target of NAB1-mediated translation control, clearly indicating that nitrosylation reduces the repressor activity of NAB1 (Figure 5-3B). This is in line with previous results (Wobbe et al., 2009) demonstrating that cysteine modification

of recombinant NAB1 either by oxidation or by alkylation *in vitro* was accompanied by a decrease in RNA binding affinity for the target mRNA sequence. Importantly, NAB1 nitrosylation could also be detected under physiological conditions, in the absence of nitrosylating agents and the degree of C226 nitrosylation was found to be negatively correlated with the light intensity used for cultivation (Figure 5-4). This could be further confirmed by analyzing the effect of an elevated to low light shift on the accumulation of a LHCBM6 protein, whose expression is driven by a constitutive promoter instead of the low light-inducible endogenous promoter. LHCBM6 accumulation following a shift to low light could be suppressed by adding a nitric oxide scavenger (Figure 5-5). While nitrosylation of NAB1 clearly requires intracellular nitric oxide production, removal of the nitroso group is coupled to cytosolic NADPH levels via a system comprising thioredoxin h1 and the NADPH-dependent thioredoxin reductase, as indicated by *in vitro* data (Figure 5-6).

In summary, our obtained results suggest the following nitrosylation based activity regulation of NAB1 in response to light quantity changes (Figure 5-7). Under low light conditions (Figure 5-7A) *C. reinhardtii* cells accumulate LHCBM proteins (Bonente et al., 2012), which is achieved by an induction of the nuclear *LHCBM* promoter (Teramoto et al., 2002; Durnford et al., 2003). NAB1 is expressed under these conditions and a significant part of the NAB1

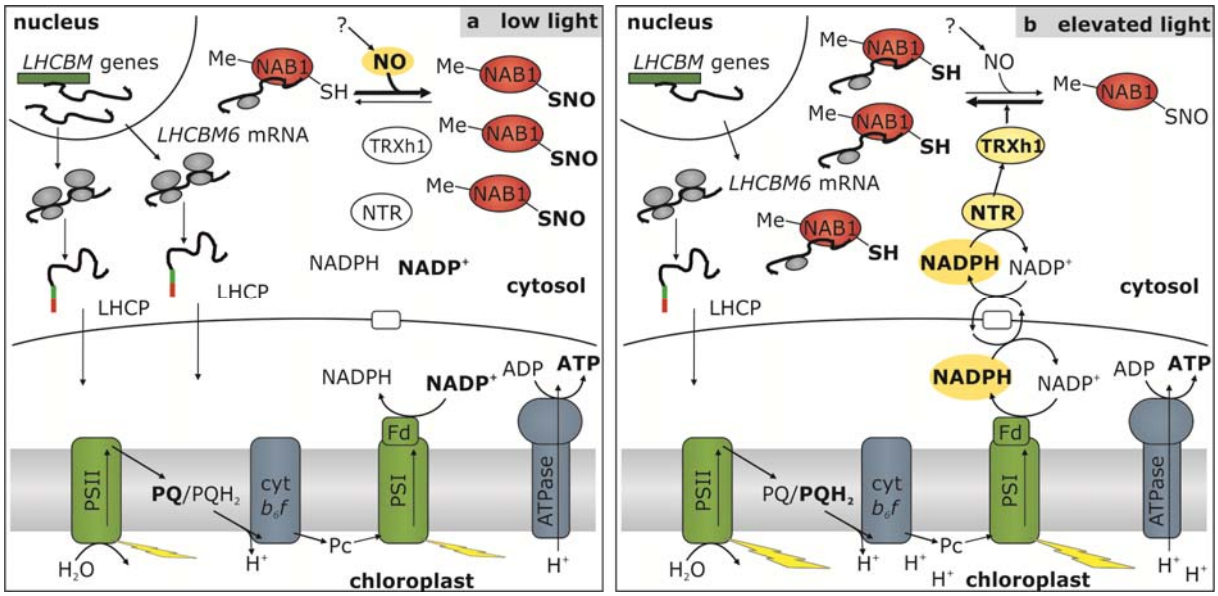

Figure 5-7 Light modulation of light-harvesting protein synthesis by nitrosylation and thioredoxin dependent denitrosylation.
Working model of the concerted LHCBM transcription and translation control under fluctuating light.
(A) Under low light the demand for light-harvesting proteins is high, which is met by high rates of nuclear *LHCBM* transcription (Teramoto et al., 2002). NAB1 is arginine methylated (Me), but a high nitrosylation level (SNO) results in a low *LHCBM* RNA binding activity, allowing accumulation of light-harvesting apoproteins (LHCP).
(B) Elevation of light intensity leads to the accumulation of reducing power (NADPH), and shuttle systems (such as the malate valve) export reducing equivalents to the cytosol. Via a system of NADPH dependent thioredoxin reductase (NTR) and thioredoxin h1 (TRX h1) this reducing power is used to denitrosylate NAB1, which activates cytosolic LHCBM translation repression. Together with a low *LHCBM* transcription (Teramoto et al., 2002), the concerted cytosolic and nuclear LHCII expression control ensures a low abundance of light-harvesting proteins when light is in excess.

population is methylated (Blifernez et al., 2011), meaning that the 'master switch' is set to the 'on' position. Nitrosylation of cysteine 226, which reduces the activity of NAB1 in low light acclimated cells, ensures that induction of the *LHCBM* promoter is not counteracted by translation repression, thus permitting the required increase in cellular LHCBM levels. Although the requirement of intracellular NO formation for NAB1 nitrosylation under low light conditions could be demonstrated in the present study (Figure 5-5B), it is currently difficult to depict the whole nitric oxide metabolism in *C. reinhardtii* (Wei et al., 2014). For the NO dependent nitrosylation in response to light limitation (Figure 5-5B), we can only rule out an implication of the reactions catalyzed by nitrate and nitrate reductase as a source of NO (Sakihama et al., 2002), since the strain used in the present study lacks a functional nitrate reductase (Hyams and Davies, 1972; Harris, 2009).

When *C. reinhardtii* cells are exposed to elevated light supply (Figure 5-7B) a bulk part of the NAB1 population is in its active state, due to the low level of nitrosylation (Figure 5-5B). In this situation, nuclear *LHCBM* promoter control and translation control work in concert to efficiently reduce the rate of LHCBM protein synthesis in cytosol. Efficient denitrosylation of NAB1 by the cytosolic thioredoxin system *in vitro* (Figure 5-6) gives an idea how the NAB1 activity state could be quickly adjusted to the prevailing light situation *in vivo*.

Light-activation of photosynthetic gene translation via cysteine based redox control is a well described process in the chloroplast of *C. reinhardtii*. An involvement of distinct thioredoxin systems, coupled either directly to the photosynthetic electron transport chain through the reduction of thioredoxin by ferredoxin-dependent enzymes (Trebitsh et al., 2000) or enzymes using NADPH as a reducing equivalent (Schwarz et al., 2012), has been proposed. Our data highlight a novel type of redox control mechanism regulating the abundance of nucleus encoded components constituting the photosynthetic apparatus in the chloroplast. A light dependent modulation of nuclear photosynthetic gene translation in the cytosol of higher plants has been demonstrated for PSI subunits (Sherameti et al., 2002), ferredoxin (Petracek et al., 1997; Petracek et al., 1998) and PSII associated light-harvesting proteins (Petracek et al., 1997) along with a requirement of photosynthetic electron transport for translational activation. These studies, however, could not provide insights into the underlying mechanisms enabling the redox cross-talk between chloroplast and cytosol.

Efficient denitrosylation of NAB1 by thioredoxin h1 is an important finding, because it exemplifies how translation of plastid targeted photosynthetic proteins is coupled to the cytosolic redox state via NADPH dependent thioredoxin reductase (Huppe et al., 1991). This thioredoxin is one two isoforms residing in the cytosol of *C. reinhardtii* cells (Lemaire and Miginiac-Maslow,

2004) and was shown to play an essential role within the alkylation induced response to DNA damage (Sarkar et al., 2005). The question arises, how alterations in the cytosolic NADPH/$NADP^+$ ratio might be connected to photosynthetic activity in the chloroplast, which is itself intimately linked to the light capture capacity at PSII. The malate oxaloacetate shuttle seems to be a reasonable candidate for such an interorganellar cross-talk (Scheibe, 2004). Under elevated light conditions, part of the NADPH produced by an increased linear electron transport activity could be used to convert oxaloacetate into malate, a form of reducing power that can shuttle between chloroplast and cytosol. A higher amount of NADPH would in turn increase the availability of reduced thioredoxin h1 required for the denitrosylation of NAB1.

Overall the proposed mechanism could quickly adjust the translation rate of an existing *LHCBM* mRNA pool to the chloroplast redox state. Perturbations of photosynthesis leading to the accumulation of reducing equivalents would transiently activate the translation repressor NAB1, while in the absence of bottlenecks downstream of photosystem I, translation of light-harvesting proteins would be permitted. NAB1 represents a key regulatory hub for the long-term adjustment of photosynthetic light harvesting capacities in the cytosol of *C. reinhardtii* cells. Details regarding the precise adjustment of NAB1 mediated translation control on various levels and in response to distinct physiological cues have been elucidated (Wobbe et al., 2009; Blifernez et al., 2011; Berger et al., 2014). Our new finding that this repressor protein is regulated via reversible nitrosylation in the cytosol opens up additional opportunities to study the molecular mechanisms underlying the intense interorganellar communication required for long-term photosynthetic acclimation in an eukaryotic cell.

Funding

This work was supported by Deutsche Forschungsgemeinschaft (KR 1586/5–2 to O.K.) and the European Union (SUNBIOPATH No245070 to O.K.),

Acknowledgements

The authors would like to acknowledge M. Hippler for providing the antibody against LHCBM6/8. We are grateful to the Center for Biotechnology (CeBiTec) at Bielefeld University for access to the Technology Platforms. No conflict of interest declared

6 Discussion

Plants and algae constantly adapt their photosynthetic apparatus to environmental changes (Anderson et al. 1995). Therefore, the adjustment of light-harvesting capacity is crucial to balance energy uptake (1.2). In the work presented here, control of light-harvesting at photosystem II upon varying light and carbon supply, as well as the interplay of short- and long-term responses were investigated, with focus on cellular regulatory hubs in the nucleus, cytosol, and chloroplast.

In the model green alga *Chlamydomonas reinhardtii*, translational regulation of nuclear encoded light-harvesting proteins (LHCBMs) is essential for unperturbed PSII antenna adjustment (Mussgnug et al., 2005). In this organism, activity of the translation repressor NAB1 was previously shown to be adjusted post-translationally by both redox based cysteine modifications and arginine methylation (Wobbe et al., 2009; Blifernez et al., 2011).

The environmental and physiological factors influencing the rate of LHCBM protein synthesis and the interrelation with short-term protective mechanisms, however, had yet to be elucidated, and these issues were addressed in this work. Here, it could be shown that NAB1 activity is fine-tuned upon moderate changes in light intensity by a mechanism implicating specific cysteine nitrosylation and thioredoxin mediated re-reduction (5). This represents a novel type of redox control that modulates protein synthesis of nuclear encoded photosynthesis associated genes in the cytosol of a photosynthetic cell. In addition to post-translational modifications, the regulation of NAB1 abundance was identified as a crucial element within the regulatory circuit adjusting PSII antenna size under fluctuating carbon dioxide supply, and control of NAB1 protein levels was based on nuclear *NAB1* promoter activity (4.2).

It was shown in this work that translational control was responsible for adjusting the level of light-harvesting proteins on longer time scales and replaced state transitions as a predominant short-term protective response (4). The cellular coordination required interorganellar cross-talk. While the light dependent regulation of NAB1 activity was based on communication between chloroplast and cytosol (5), cellular NAB1 levels under fluctuating carbon supply were controlled by chloroplast to nucleus retrograde signaling (4). To analyze *cis*-regulatory sequences on the nuclear *NAB1* promoter, a reporter system was developed which exploits efficient secretion of a reporter protein (3). Applying this system, a short promoter sequence was identified that is sufficient to confer responsiveness to carbon dioxide limitation and harbors hitherto unknown transcriptional regulatory elements (4.3).

In the following sections, novel findings are discussed with a focus on the applicability of the reporter system developed to study nuclear encoded photosynthesis associated genes (6.1), the signaling pathways involved in LHCBM translation control (6.2) and the interrelation of short- and long-term mechanisms regulating light-harvesting at photosystem II (6.3).

The results obtained within this thesis are integrated into a model (6.4) depicting strategies of *C. reinhardtii* to control PSII excitation pressure under different physiological conditions, and the general relevance of this model for light-harvesting regulation in phototrophic eukaryotes is evaluated.

6.1 A versatile *Gaussia* luciferase reporter system exploiting efficient secretion facilitates the analysis of nuclear *C. reinhardtii* promoters

Reporter systems greatly ease promoter analysis (1.3.3). As a prerequisite, the reporting protein needs to release a signal that is easy to detect, linear to protein amount, and mirrors transcriptional activity of the promoter driving expression of the reporter gene (Hallmann, 2007). The *Gaussia* luciferase (*g*Luc) used in this work exhibits all these characteristics, emitting a very bright luminesce signal that correlates with protein concentration over five orders of magnitude (Verhaegen and Christopoulos, 2002; Shao and Bock, 2008; 1.3.3). Furthermore it was shown only recently that protein accumulation follows RNA levels with a delay of only thirty minutes in *Volvox carteri* (von der Heyde et al., 2015), allowing near real-time detection of promoter activity.

The *g*Luc gene encodes a secretion signal that promotes protein export via the endoplasmic reticulum and Golgi apparatus in *C. reinhardtii*, leading to protein accumulation in the culture supernatant of cell wall deficient strains (Ruecker et al., 2008). As the signal peptide was not optimized for use in algae in previous studies, replacement of the native secretion signal with a *C. reinhardtii* signal peptide derived from the highly expressed periplasmic carbonic anhydrase 1 was assumed to enhance protein export (3.2). Indeed, although the same promoter drove *g*Luc transcription, transformant algae using the native *C. reinhardtii* signal exhibited a six times higher luminescence signal (Figures 3-3 and 3-4). As the luciferase signal clearly stood out from any background signal, the enhanced secretion facilitated screening of transformant cell lines and quantitative promoter analysis (Figures 4-2 and 4-12).

Expression of carbonic anhydrase 1 is dramatically increased under carbon dioxide limitation, but information about the regulation of secretion itself is rare (Fukuzawa et al., 1990; Spalding, 2009; 6.2.1). To rule out any effect of the secretion signal applied on the subsequent promoter studies under fluctuating carbon availability, transformant cell lines were generated in which a promoter-less luciferase gene was integrated into the genome and expressed from randomly trapped promoters (4.3.2). None of the strains investigated showed a differential luminescence signal under low compared to high carbon dioxide supply (Figure 4-12), indicating that promoter activity rather than post-transcriptional mechanisms, including secretion, determined reporter protein accumulation in the culture supernatant.

Importantly, the luminescence signal indeed directly correlated with the amount of protein and transcript of the gene of interest (Figures 3-4 and 4-2). The improved reporter system could therefore be used to study carbon dependent expression of the LHCBM translation repressor NAB1 and to identify regulatory elements on the *NAB1* promoter (6.2.1).

6.2 LHCBM translational control is at the crossroads of retrograde signaling pathways

Light-harvesting capacity is regulated at multiple levels with responses occurring over a range of time, and control of LHCBM protein synthesis results in changes in antenna size at photosystem II in the long term (1.2.3; Figure 6-2A). The cytosolic LHCII translation repressor NAB1 is a key component of these adjustment mechanisms in *C. reinhardtii* (Mussgnug et al., 2005; Wobbe et al., 2009; Blifernez et al., 2011; 1.2.3). Post-translational modifications were shown to control the RNA binding activity of NAB1, including redox based cysteine modification and arginine methylation (Figure 1-5). Methylation of two arginine residues in the glycine arginine rich motif I has slow turnover rates and is costly regarding ATP input (Fackelmayer, 2005), and was therefore regarded as a less dynamic 'master switch' which adjusts NAB1 activity to current growth conditions (Blifernez et al., 2011). In contrast, cellular redox states may change rapidly, and redox based modification of NAB1 cysteine residues, which fine-tunes repressor activity, can be regarded as fast response dynamically regulating light-harvesting capacities (Wobbe et al., 2009). The trigger, the signaling pathway, and the mechanism of this redox control, however, remained to be elucidated.

In this thesis, details of the mechanism behind redox based cysteine modification of NAB1 were elucidated (5). A system involving specific single cysteine nitrosylation (Figure 6-1, NAB1-SNO, low light) and probably thioredoxin mediated reduction (Figure 6-1, NAB1-SH, elevated light) adjusted NAB1 activity to the demand for light-harvesting antenna proteins in response to moderate changes in light intensity.

The control of NAB1 expression could be shown for the first time in this work (4.2; 4.3.2). Carbon dioxide limitation induced or de-repressed *NAB1* transcription in the nucleus, eventually adjusting the accumulation of light-harvesting proteins in response to the current carbon assimilation mode. Impaired NAB1 expression in a state transitions mutant and in response to photosynthetic electron transport inhibition indicated that retrograde signals emerging from the chloroplast control nuclear *NAB1* promoter activity (Figure 6-1, dashed arrows).

Retrograde signaling pathways regulating nuclear photosynthetic gene expression were shown to converge (Koussevitzky et al., 2007). However regarding the different levels of NAB1 regulation in *C. reinhardtii*, the signal transduction and perception mechanisms seemingly differ or diverge. Both carbon deprivation and the elevation of light intensity cause an over-reduction of the photosynthetic electron transport chain and raise excitation pressure on photosystem II (1.2; Figure 6-2B). However, while CO_2 limitation triggered an increased NAB1 accumulation based on nuclear promoter activity (Figures 4-2 and 4-3), this was not the case under elevated light

intensity (Figure 5-5). In contrast, changes in light intensity modulated the activity of NAB1 in the cytosol (Figures 5-4 and 5-5). These differences indicate that carbon and light supply are sensed and communicated on distinct pathways within the cell.

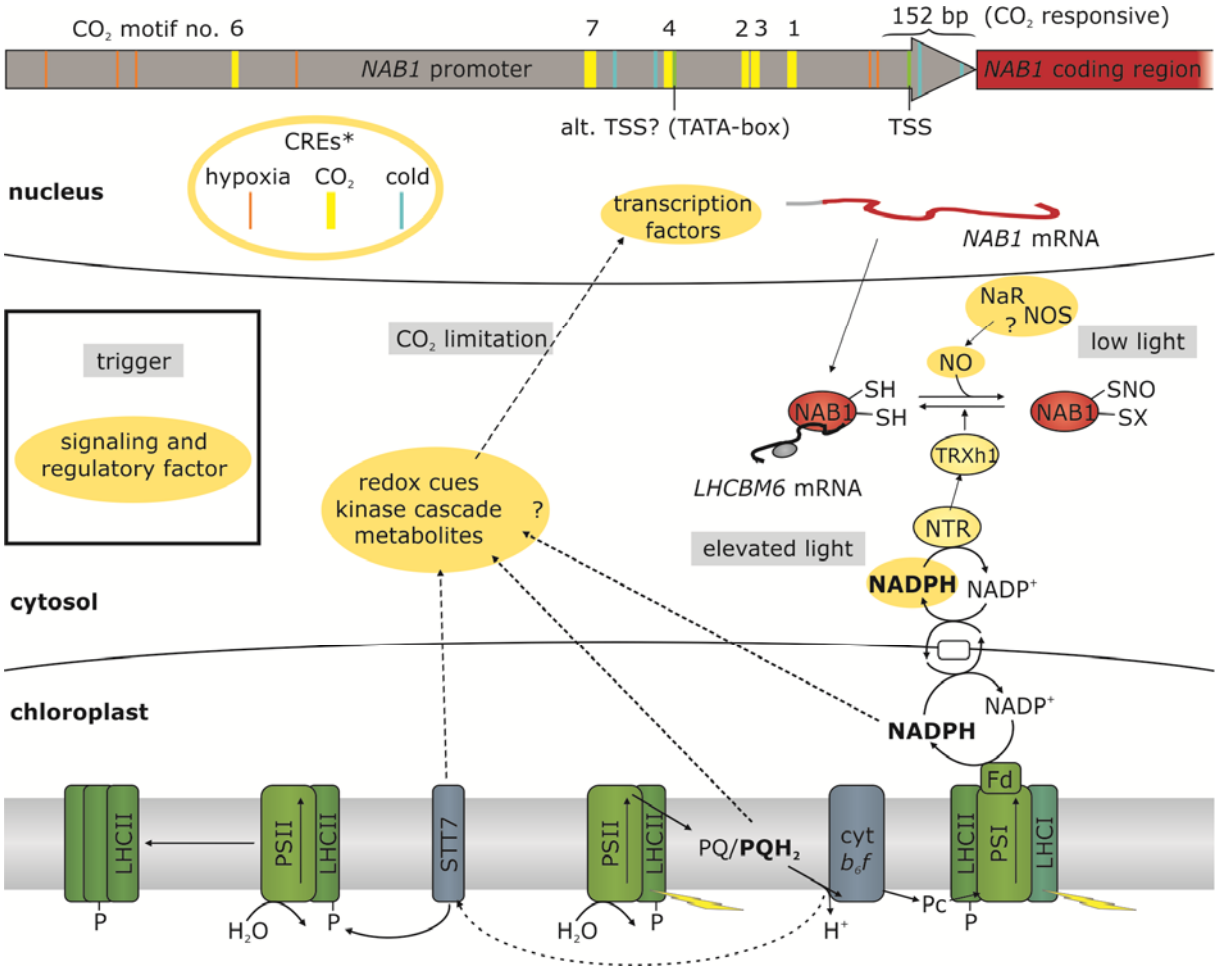

Figure 6-1 Multi-compartmental regulation of the LHCBM translation repressor NAB1.
Working model depicting retrograde signaling pathways (dashed arrows) controlling LHCBM protein synthesis. The communication towards the nucleus under carbon dioxide limitation is discussed in 6.2.1 and towards the cytosol under fluctuating light in 6.2.2. (*) Putative CREs revealed via *in silico* studies, CO_2 motifs as previously identified (Winck et al., 2013a; Table 4-2). (alt.) TSS: (alternative) transcription start site; CREs: *cis*-regulatory elements; NaR: nitrate reductase; NOS: nitric oxide synthase; NTR: NADPH dependent thioredoxin reductase; TRX h1: thioredoxin h1; further abbreviations as described (1).

The regulation of NAB1 mediated LHCBM translation repression via the modulation of nuclear *NAB1* promoter activity (6.2.1) and redox based cysteine modification in the cytosol (6.2.2) are discussed in the following sections in the context of signaling pathways.

6.2.1 Regulation of the *NAB1* promoter involves chloroplast to nucleus signaling

LHCBM translational repression efficiently reduced the biochemical and functional photosystem II antenna size, thus relieving PSII excitation pressure under carbon dioxide limitation (4.2). Accumulation of the translational repressor NAB1 emerged as key element of this response, and analysis of the state transition mutant *stt7*, as well as application of an inhibitor of photosynthetic electron transport (Figure 4-6), indicated that signals originating in the chloroplast control nuclear *NAB1* transcription (Figure 6-1, dashed arrows).

In order to gain insight into how these signals are implemented in the nucleus, the structure of the *NAB1* promoter was investigated and the position of regulatory sequences was systematically narrowed down (4.3.2). The transcription start site was determined to be located 102 bp upstream of the translation start for cells during late-logarithmic photoheterotrophic growth (Figure 6-1, TSS), similar to the length of 5' untranslated regions of other nuclear *C. reinhardtii* genes, for instance *HSP70A* (von Gromoff et al., 2006) and *LHCBM6* (Hahn and Kück, 1999). The existence of a second transcription start under different conditions is, however, not excluded. Transcription starting at two alternative sites, one of which encoding a TATA-box, was reported for other nuclear genes of *C. reinhardtii* before (von Gromoff et al., 2006; Fischer et al., 2009; 1.3.3). Indeed, a sequence between -483 and -478 bp upstream of the NAB1 translation start resembles a TATA-box and the region -377 to -358 bp is adenine thymine rich (Table 4-2); both elements might indicate an alternative transcription start (Figure 6-1, alt. TSS).

The reporter system developed (3.2; 4.2; 6.1) was used to narrow down *NAB1* promoter elements that confer responsiveness to carbon dioxide limitation (4.3.2). Five constructs with truncated promoter versions were introduced into wild-type *C. reinhardtii* cells and three independent cell lines per construct were tested regarding carbon dioxide dependent transcriptional activity. All strains showed an increased luminescence signal under CO_2 limitation, in contrast to the control strains harboring a promoter-less luciferase construct (Figure 4-12). The cell lines expressing luciferase from a *NAB1* promoter sequence 152 bp upstream of the translation start, the smallest fragment tested, exhibited an on average three to four times higher luminescence under low compared to high carbon dioxide supply (4.3.2). In conclusion, this fragment comprises essential sequences for both basal and differential transcription, and could be further investigated in detail to identify *cis*-regulatory elements and to find transcription factors involved in CO_2 dependent promoter activity (7).

A transcriptome study, independent of the work presented here, confirms the increased expression of NAB1 under carbon dioxide limitation (Winck et al., 2013a). In that study, candidate regulatory elements were identified at genome scale and a regulatory network of

transcription factors and regulators was constructed. *NAB1* was found among the early responsive genes, with a seven-fold increased mRNA amount within one hour, eleven-fold after two and three-fold after three hours of carbon dioxide limitation in photoautotrophic conditions. The fast response may indicate that preexisting signaling pathways are used (Dietz, 2015), which requires further consideration in future experiments designed to identify the transcription factors involved (7).

In the same study, ten putative regulatory motifs and motif combinations in promoter regions of genes with enhanced expression under low carbon dioxide were identified (Winck et al., 2013a), and six of these motifs are present in the NAB1 promoter (Table 4-2; Figure 6-1, CREs, yellow lines, motifs 1 to 4, 6, 7). However, none of them are located in the sequence 152 bp upstream of translation start. As this fragment is sufficient to confer CO_2 responsive transcription (Figure 4-12), this indicates that additional, hitherto unknown regulatory elements are encoded in this sequence.

The acclimation to inorganic carbon has been extensively studied in *C. reinhardtii*, including the response of photosynthetic reactions, dissipative pathways and the carbon concentrating mechanism, which increases the concentration of CO_2 in the vicinity of RuBisCO via a system of carbonic anhydrases and hydrogen carbonate transporters upon carbon dioxide limitation (Spalding, 2009; Wang et al., 2015; 1.1.2). However, the signaling molecule(s) and receptor(s) are not unambiguously identified, and a direct sensing of the concentration of inorganic carbon species as well as indirect cues such as photosynthetic or photorespiratory metabolites and redox poise were suggested (Spalding and Ogren, 1982; Ramazanov and Cardenas, 1992; Villarejo et al., 1997).

Despite the uncertainty about the initial signal, some factors involved in the regulatory pathway inducing (or de-repressing) the carbon concentrating mechanism in *C. reinhardtii* are known (Wang et al., 2015), with the transcription factor CCM1, also termed CIA5, identified as 'master regulator' (Fukuzawa et al., 2001; Xiang et al., 2001). This constitutively expressed protein activates either directly or indirectly expression of further regulatory proteins such as the MYB-like transcription factor LCR1 (Yoshioka et al., 2004). LCR1 in turn controls transcription of genes involved in the carbon concentrating mechanism, and was particularly shown to bind to enhancer elements of the carbonic anhydrase 1 promoter to induce or maintain *CAH1* transcription under low CO_2 supply (Kucho et al., 1999; Kucho et al., 2003; Yoshioka et al., 2004).

Regulation of *NAB1* transcription via CCM1 and LCR1, however, does not seem probable. Database searches revealed that the enhancer consensus sequences of the *CAH1* promoter are

present five times on the reverse strand of the *NAB1* promoter (4.3.2), but the kinetics of *NAB1* and *LCR1* transcript accumulation upon carbon dioxide limitation differ clearly (Winck et al., 2013a). In a transcriptome study comparing gene expression between a wild-type strain and a mutant lacking CCM1, there was no difference in *NAB1* mRNA abundance under high CO_2 supply in between strains, and a 1.5-fold higher level in the mutant under carbon dioxide limitation (Fang et al., 2012). If CCM1 positively regulated *NAB1* transcription, the opposite and a much stronger difference in between strains would be expected under low CO_2 conditions. For example, the transcript level of the carbonic anhydrase 1 was more than 500-fold lower in the knock out mutant under carbon dioxide limitation compared to wild-type cells (Fang et al., 2012). The slightly increased *NAB1* mRNA level in the mutant cells could be due to a more severe shortage of inorganic carbon, as the carbon concentrating mechanism is impaired. As yet another difference, acetate availability prevents the induction of the carbon concentrating mechanisms (Fett and Coleman, 1994), but NAB1 expression is further increased if organic carbon as acetate is supplied (4.2).

The nearly unaltered NAB1 expression in the CCM1 knock out mutant compared to the wild-type under carbon dioxide limitation, the different kinetics of *NAB1* and *LCR1* transcript abundance increase, and its inducibility in acetate containing media suggest that *NAB1* transcription is not controlled via the 'master regulator' pathway.

The experiments performed in this work indicate that, rather than concentration of inorganic carbon *per se*, symptoms of carbon dioxide deprivation, such as metabolic changes or the over-reduction of the photosynthetic electron transport chain are involved in the regulation of *NAB1* transcription (Figure 6-1, dashed arrows). Both the inhibition of photosynthetic electron transport as well as a lack of the state transition kinase impaired NAB1 accumulation during a switch from inorganic to organic carbon consumption (Figure 4-6), suggesting that signals originating in the chloroplast control the nuclear *NAB1* promoter.

Other environmental situations are known to cause similar symptoms, and *in silico* database searches revealed further candidate *cis*-regulatory elements on the *NAB1* promoter (4.3.2; Table 4-2). Six sequences responsive to copper and oxygen limitation in *C. reinhardtii* (Quinn et al., 2002; Kropat et al., 2005; Figure 6-1, CREs, orange lines) and four sequences involved in acclimation responses to low temperature in vascular plants (Jiang et al., 1996; Dunn et al., 1998; Kim et al., 2002; Figure 6-1, CREs, blue lines) are present on the *NAB1* promoter. A reduction of photosystem II antenna size is reasonable under both hypoxic and cold conditions. A decrease in temperature lowers the rate of enzymatic reactions. In consequence, metabolic reactions including the Calvin cycle are slowed down, but physical processes such as harvesting of light

energy and transfer of excitons proceed. This results in an over-excitation of the photosystems. Oxygen limitation decreases the consumption of reducing equivalents in the mitochondrial electron transport chain (1.1.1), which feeds back into photosynthetic electron transport due to a slower regeneration of electron acceptors and because electrons may be transferred back to plastoquinone (1.1.1). The resulting over-reduction of the PQ-pool triggers a state II transition (1.2.2). Similar to observations under carbon dioxide limitation, a reduction of PSII antenna size through NAB1 mediated LHCBM translation repression could efficiently relieve PSII excitation pressure upon hypoxia or cold in the long term.

In contrast to the environments examined above, conditions triggering oxidative stress decreased NAB1 expression. A ten-fold increase in light intensity to 1000 µmol photons m^{-2} s^{-1} under a photoautotrophic, low carbon dioxide growth regime leads to the generation of reactive oxygen species which trigger a global polysome assembly (McKim and Durnford, 2006). The same condition reduced the NAB1 transcript and, with a delay, protein levels in this work (Figure 4-10). The low abundance of NAB1 suggests that specific translation repression mediated by NAB1 is not the cause of reduced light-harvesting protein levels under this condition. Rather the general polysome disassembly reported accounts for the post-transcriptional regulation of LHCBM expression (Durnford et al., 2003; McKim and Durnford, 2006). Furthermore, application of the PET inhibitor DCMU led to a rapid and strong decrease of NAB1 protein levels (Figure 4-6). This herbicide blocks the plastoquinone binding site of PSII and therefore causes the oxidation of both intersystem and soluble stromal electron carriers, which inactivates STT7, and under certain conditions increases the generation of reactive oxygen species (Fufezan et al., 2002; He and Häder, 2002). PET interruption is a severe intervention and consequent impairments are not always easy to disentangle (Trebst, 2007). However, together with the low NAB1 abundance under high light stress, this result further points towards the importance of reducing power and/or an active state transition kinase for *NAB1* transcription.

To summarize, an increased NAB1 expression based on nuclear promoter activity results in an efficient reduction of PSII antenna size under prolonged carbon dioxide limitation. A 152 bp *NAB1* promoter sequence, including the 102 bp long 5'UTR, is sufficient to drive CO_2 dependent transcription, and contains hitherto unknown *cis*-regulatory elements in addition to previously identified motifs further upstream.

Regulation of NAB1 likely occurs independent of the 'master regulator' of the carbon concentrating mechanism, CCM1, and is controlled by signals emerging from the chloroplast. In general, conditions favoring reduced states of electron carriers correlate with an increased NAB1

expression, while oxidizing, ROS generating conditions, such as high light stress, are associated with low NAB1 levels.

Similar to carbon dioxide limitation, hypoxia and low temperatures cause an over-reduction of the photosynthetic electron transport chain, and candidate regulatory elements involved in acclimation responses to oxygen limitation and low temperatures could be identified on the *NAB1* promoter. Whether NAB1 transcription actually differs under these conditions needs to be revealed experimentally, and the *NAB1*::*g*Luc reporter system developed in this work (3; 4; 6.1) can be used to this end (7).

6.2.2 Regulation of NAB1 activity involves chloroplast to cytosol signaling

The cellular redox poise modulates the accumulation of LHCII (1.2.3; 1.3), and activity of the LHCBM translation repressor NAB1 is fine-tuned through redox based modification of cysteine residues (Wobbe et al., 2009; 1.2.3). Two cysteines at amino acid position 181 and 226 in the C-terminal RNA recognition motif are involved in this regulation (Figure 1-5). In the thiol state, NAB1 binds to *LHCBM* transcripts and therewith represses translation, whereas modification of cysteines through alkylation or oxidation leads to a low RNA binding activity (Wobbe et al., 2009). Replacement of either cysteine residue with serine mimics a permanently active state of NAB1. *C. reinhardtii* mutants expressing such modified versions of NAB1 therefore display a pale green, small antenna phenotype, and replacement of C226 or double mutation causes a much stronger phenotype than replacement of C181 alone (Wobbe et al., 2009; Figure 1-5).

However, the physiological trigger of this redox modification and the factors involved were hitherto unknown. The formation of intramolecular disulphide bonds was ruled out previously through peptide mapping analyses (Wobbe et al., 2009), indicating that reversible modification via thiol reactive molecules could be implicated in this control. In the study presented here (5.2), a combination of mass spectrometry, the biotin switch technique (Jaffrey and Snyder, 2001) and monitoring of LHCBM protein levels revealed nitrosylation of NAB1 at C226 *in vivo* under low light conditions of 40 µmol photons m^{-2} s^{-1} (Figures 5-2 to 5-5; Figure 6-1, NAB1-SNO, low light). This modification lowered NAB1 activity and thus allowed translation of LHCBM proteins to ensure efficient photosynthetic light capture when light is limiting. NAB1 denitrosylation occurred under elevated light of 200 µmol photons m^{-2} s^{-1} (Figure 5-4 and 5-5; Figure 6-1, NAB1-SH, elevated light), which increased LHCBM translation repression and therewith reduced the surface for light absorption.

In vitro studies (Figure 5-6) strongly suggest that NAB1 reduction is catalyzed by the cytosolic thioredoxin h1 (Figure 6-1, TRX h1) and NADPH dependent thioredoxin reductase (Figure 6-1,

NTR). A signaling pathway from photosynthetic electron transport in the chloroplast to LHCBM translation repression in the cytosol via export of plastidic reducing equivalents therefore seems plausible. Under elevated light, the increased energy absorption and transfer leads to an accumulation of the reducing equivalent NADPH downstream of photosystem I (Figure 6-1, NADPH). Excess reducing equivalents are exported from the chloroplast via shuttles like the malate/oxaloacetate transport system (Hoefnagel et al., 1998; Figure 1-2, MAL/OAA). Such "valves" therewith fulfill a dual function, as they both relieve plastidic electron pressure and signalize over-reduction of the chloroplast to the other compartments (Foyer and Noctor, 2009). In the cytosol, electrons are reshuffled from NADPH to NAB1 cysteines via TRX h1 and NTR, which implements the redox signal by decreasing the synthesis of light-harvesting proteins.

The regulation of NAB1 activity via nitrosylation and thioredoxin mediated reduction is the first detailed description of redox based translation control of nuclear encoded photosynthesis associated genes. Translation regulation is the main mechanism regulating gene expression in the chloroplast (Wobbe et al., 2008), and plastidic RNA binding proteins such as RB47 and the large subunit of RuBisCO were shown to be regulated via cysteine modification (Alergand et al., 2006; Cohen et al., 2006). In these systems, reducing equivalents from photosynthetic electron transport are directly used to modify translation factor activity. In the case of cytosolic translation control on LHCBM proteins, signaling molecules need to be translocated between compartments, and the mechanism depicted here provides new insights into how retrograde signals are transduced and exert effects on light-harvesting capacities.

Considering the moderate light intensity variations applied in this study, NADPH seems reasonable as a signaling cue, since the redox state of NADPH/$NADP^+$ is directly affected by photosynthetic electron transport (1.1.1, Figure 1-2), while other reductants such as glutathione and ascorbate are generally kept in a highly reduced state under non-stress conditions (Foyer and Noctor, 2009; 1.3.1). Intriguingly, increasing $NADP^+$ concentrations inhibit the activity of malate dehydrogenase (Scheibe and Jacquot, 1983) which uses NADPH to reduce oxaloacetate to malate. This allows a double control via substrate availability and product feedback inhibition, which strengthens the importance of a strict control on redox shuttling systems between organelles.

In low light conditions, NAB1 was clearly nitrosylated at C226 (Figure 5-4), but the source of nitric oxide remains to be revealed (Figure 6-1). Generation of nitric oxide involving nitric oxide synthase (NOS) similar to mammalian enzymes and nitrate reductase (NaR) were considered in *C. reinhardtii* (Wei et al., 2014; 1.3.1). Sakihama et al. (2002) suggested that NO is produced as unwanted byproduct by NaR when electron transport in inhibited, while NOS catalyzes a highly

regulated reaction, with controlled levels of nitric oxide serving as signaling molecule. In the study presented here, NaR deficient algal strains were used (5.2). Nevertheless, nitrosylation of NAB1 was observed under low light intensity, indicating that the presence of NaR is not a prerequisite for nitric oxide generation under the conditions examined. It is however possible that nitric oxide mediated regulation of NAB1 is stronger in a NaR expressing strain, which could be considered in subsequent studies (7).

The interplay of S-nitrosylation and thioredoxin mediated denitrosylation has been intensively studied in animal cells, and target proteins are involved in cytoskeletal organization, cellular metabolism, signal transduction, and redox homeostasis (Benhar et al., 2008; Benhar et al., 2010). In plants, nitrosylation and TRX h5 mediated re-reduction are essential for plant immune responses (Tada et al., 2008; Kneeshaw et al., 2014). The reduction of S-nitrosothiols as described by these studies and the work presented here extends the substrate repertoire of the NTR/TRX system from its well-known disulphide forming substrates to protein-SNO targets. In a recent study nearly 500 S-nitrosylated proteins were found in *C. reinhardtii*, many of them involved in photosynthesis, but also carbohydrate metabolism, amino acid metabolism, translation, protein folding or degradation, cell motility, and stress (Morisse et al., 2014). Novel targets of thioredoxins were revealed in higher plants and animal cells using affinity chromatography and quantitative proteomics (Yamazaki et al., 2004; Benhar et al., 2010), and similar techniques can be applied in future research to identify nitrosylated proteins that are reduced by thioredoxins in *C. reinhardtii.*

Overall, this work describes the mechanism of redox dependent control on light-harvesting protein synthesis. Excess reducing power is sensed via NAB1 denitrosylation in the cytosol under elevated light and adjusts the accumulation of LHCBM proteins to the demand in the photosynthetic electron transport chain. Observations in higher plants suggests that redox control on LHCII abundance occurs on post-transcriptional rather transcriptional level (Frigerio et al., 2007; 1.2.3), which is reasonable as redox signaling occurs rapidly and requires fast responses, and translation control provides a much faster adjustment of protein levels than transcription control (Dietz, 2015). The results presented in previous work (Wobbe et al., 2009) and here (5) on redox based and stimulus dependent cysteine (de-)nitrosylation of the translation repressor NAB1 support the conclusion that retrograde redox signals modulate the level of LHCBM protein synthesis to adjust light-harvesting at photosystem II.

6.3 Short- and long-term light-harvesting regulation – Revisited

Plants and algae continually adjust their photosynthetic apparatus to environmental conditions (1.2). While PSII core structure and assembly is highly conserved throughout evolutionary lineages (Nickelsen and Rengstl, 2013), the outer antenna and the regulation of light-harvesting differ substantially (Büchel, 2015; Ruban, 2015), indicating that controlling light-harvesting capacity is the most feasible strategy to cope with the specific environmental circumstances.

Some general mechanisms can be outlined that regulate light-harvesting on different time scales (Figure 6-2A). Energy dependent quenching (qE) is induced and reverted quickly within seconds to minutes and state transitions (qT) operate on time scales of minutes to hours (1.2.2). Within long-term mechanisms, translation control shows a faster response on protein levels than transcription control (Dietz, 2015; 1.2.3; 6.2.2). Under severe stress, reactive oxygen species evolve, which trigger a global polysome disassembly (McKim and Durnford, 2006; Floris et al., 2013) and cause photoinhibition (qI) due to PSII damage occurring faster than its repair (Niyogi, 2009; 1.2.2).

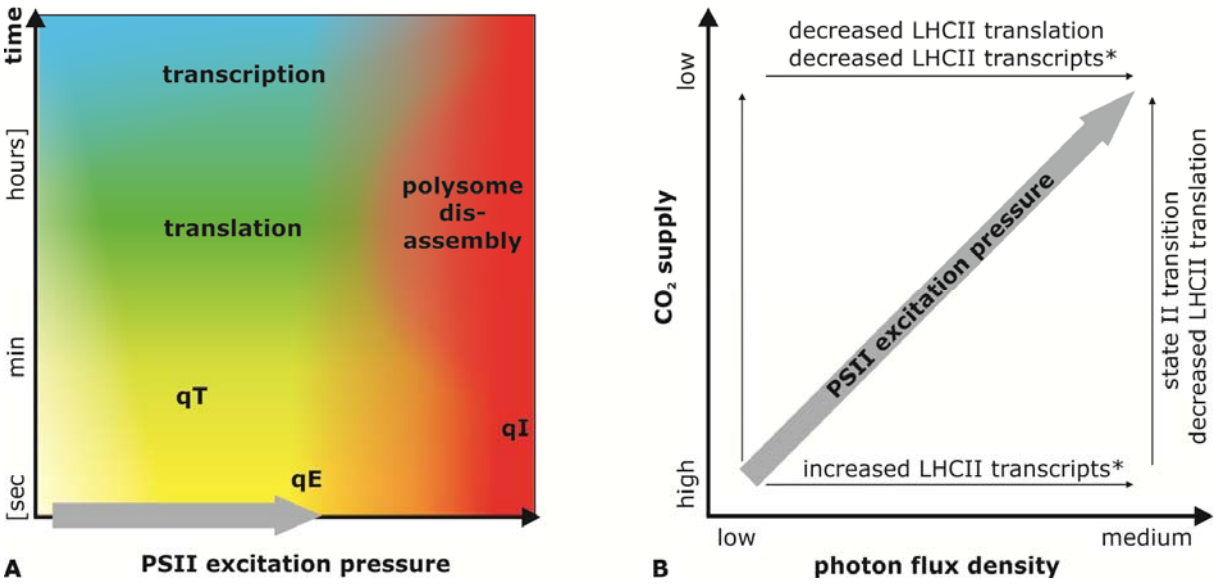

Figure 6-2 Photoprotective and regulatory reponses adjust light-harvesting at PSII over a range of envionmental triggers and time.
(A) Action time of responses regulating light-harvesting at PSII. qE: energy dependent quenching; qT: state transitions; qI: photoinhibition. (B) PSII excitation pressure under physiological conditions without oxidative stress (grey arrow in A and B) caused by varying carbon dioxide and/or light supply triggers specific regulatory responses. Transcript levels of LHCBM (*) were analyzed previously (Teramoto et al., 2002).

This general scheme is true for plants, mosses and green alga investigated so far. However, as indicated above, every photosynthetic organism developed own strategies to cope with its specific environment. Photo-protective responses in the green alga *C. reinhardtii* have been intensively studied over decades, and ongoing research still provides new insights. This thesis

aimed to elucidate novel aspects in the interrelation of short- and long-term regulation of light-harvesting regulation. The findings presented in the chapters 4 and 5 are discussed here against the background of current debates.

6.3.1 The role of state transitions in modulating light capture

State transitions were originally described as redistribution of light harvesting-complexes under PSII and PSI specific light (Bonaventura and Myers, 1969; Murata, 1969). Since then, many studies investigated the role of qT type quenching under numerous conditions such as high light and carbon dioxide deprivation (Falk and Palmqvist, 1992; Iwai et al., 2007; Allorent et al., 2013) and details of the underlying mechanism and regulation were elucidated (1.2.2). There is general consensus that unphosphorylated LHCII stabilize the photosystem II megacomplex in state I (1.2.2; 6.3.2). Phosphorylation of certain subunits triggers megacomplex dissociation and detachment of antenna complexes in state II, and the remaining C_2S_2 and C_2 type PSII have a greatly reduced absorption cross-section (1.2.2; 6.3.2).

However, the extent of LHCII associated to photosystem I under state II as well as the interrelation of state transitions and cyclic electron flow (Finazzi et al., 2002; Lucker and Kramer, 2013; Takahashi et al., 2013) are currently debated. A photo-acoustic study long dominated the view that while in plants approximately 15 to 20% of LHCII bind to PSI (Allen, 1992), this portion is much larger with up to 80% in *C. reinhardtii* (Delosme et al., 1996). Recent studies challenged this interpretation. A combination of absorption spectroscopy and chlorophyll *a* fluorescence measurements revealed that 70% of LHCII detach from PSII, while only 20% bind to PSII (Nagy et al., 2014). Similarly, applying low temperature chlorophyll a fluorescence emission spectra and time resolved fluorescence decay, Ünlü et al. (2014) determined that LHCII dissociation involves up to 80% of the PSII antenna, but only 10% of the released complexes bind to PSI. These findings could be reasonable as the light-harvesting antenna of PSI is already very large in *C. reinhardtii* (Kargul et al., 2003; Drop et al., 2011) and highly efficient (Le Quiniou et al., 2015). Higher plants have much smaller PSI antenna, with four instead of nine LHCI monomers as in the green alga, so that the binding of LHCII trimer(s) induces a significant change in the absorption cross-section (Minagawa, 2011; Kouril et al., 2012; Drop et al., 2014b).

Conversely, one could reason that because of a large PSI antenna in *C. reinhardtii*, binding of more LHCII complexes are necessary to achieve a substantial increase in absorption cross section. Intriguingly, although applying the same methods as Ünlü et al. (see above), Wlodarczyk et al. (2015) recently found that two thirds of the released light-harvesting antenna transfer energy to PSI, while the remaining third is probably in a quenched state. The difference might be explained

by a different sample treatment, as the latter authors locked the state of the cells chemically with sodium fluoride, which inhibits dephosphorylation and prevents unwanted state transition relaxation (Telfer et al., 1983).

Despite discrepancies in the postulated amount of LHCII associated with PSI under state II conditions, all recent studies imply the existence of a pool of free LHCII that may function in dissipating excess energy (Nagy et al., 2014; Ünlü et al., 2014; Wlodarczyk et al., 2015). In fact, aggregates of free LHCII, similar to those observed under high light stress related energy depending quenching, have been observed under classical state II conditions (Betterle et al., 2009; Ruban and Johnson, 2009; Tokutsu et al., 2009; Iwai et al., 2010).

The concordantly observed large portion of LHCII dissociating from PSII in state II, the formation of aggregates and the interplay of qT and qE (Allorent et al., 2013; 1.2.2) support the view that a major physiological function of state transition is the photo-protective reduction of the absorption cross-section at PSII in *C. reinhardtii*. Indeed, high energy quenching, the major NPQ process in plants, is generally comparatively low in *C. reinhardtii* (Finazzi et al., 2006) and very much depends on the growth condition (Peers et al., 2009). In plants and green algae, qE involves the protonation of LHC-like proteins and their binding to LHCII (Tokutsu and Minagawa, 2013; Xue et al., 2015; 1.2.2), but the mechanisms differ in *A. thaliana* and *C. reinhardtii*. The four-helix protein PSBS involved in qE in *A. thaliana* does apparently not bind pigments and is constitutively expressed (Niyogi and Truong, 2013). In contrast, expression of the pigmented LHCSR3 of *C. reinhardtii* is induced under stress conditions, presumably to prevent energy loss under limited light supply through unintended quenching by bound xanthophylls (Peers et al., 2009; Niyogi and Truong, 2013). While expression of LHCSR3 is in progress and qE not fully operating, other fast protective mechanisms including state transitions are hence important in this alga. Consistently, qT is a major part of NPQ in *C. reinhardtii* acclimated to low light (Finazzi et al., 2006).

In this work, CO_2 limitation and simultaneous acetate supply induced a state II transition in *C. reinhardtii* (Figures 4-6 and 4-8), in agreement with previous studies under photoautotrophic conditions (Palmqvist et al., 1990; Falk and Palmqvist, 1992; Iwai et al., 2007). Carbon dioxide deprivation leads to an over-reduced photosynthetic electron transport chain and raises the demand for ATP, an imbalance that is further increased by acetate availability (1.1.2). A state II transition could fulfill a dual function under this condition, to relieve of PSII excitation pressure and to adjust the ratio of ATP to NADPH through enhancing cyclic electron flow (1.1.1). Intriguingly, wild-type cells showed a higher electrochromic shift signal under carbon dioxide limitation (Figure 4-8), pointing towards an increased cyclic electron flow activity. However, this

enhanced activity was detected twelve hours after the onset of the limitation, when state transitions were already relaxed (Figures 4-6 and 4-8), indicating that cyclic electron flow occurs independently of qT under low carbon dioxide supply in the long term, similar to the independent regulation in response to forty minutes of dark and anoxic conditions described before (Takahashi et al., 2013).

The dissociation of a major fraction of LHCII from PSII and the presence of a free LHCII pool in a quenching mode in state II, in line with an independence of qT and cyclic electron flow and a generally low capacity for qE emphasize the dominant role of state transitions as short-term PSII protective mechanism in *C. reinhardtii*. Interestingly, the expression of LHCSR3 was shown to be induced under carbon dioxide limitation as well (Yamano et al., 2008; Peers et al., 2009), and binding of this complex to detached LHCII is thought to enable energy dissipation under state II conditions (Niyogi and Truong, 2013). As evidence for mechanistic similarities of qE and qT is growing (Erickson et al., 2015; 1.2.2), the interrelation of both NPQ mechanisms will be an interesting topic in future research (7).

6.3.2 Isoform specific LHCII translation control within acclimation responses

Control on light-harvesting protein biosynthesis emerged as central element of long-term photosynthetic acclimation responses upon varying carbon and light supply (4; 5; 6.2). The abundance of different types of LHCII appeared co-regulated and depended on the activity of the translation repressor NAB1 in the work presented here (4.2), in agreement with previous studies (Wobbe et al., 2009; Blifernez et al., 2011). The question arises, how the control of LHCII protein levels is concerted, although NAB1 shows a strong preference towards the mRNA of isoform *LHCBM6* (Mussgnug et al., 2005). Co-immunoprecipitation studies revealed a binding to the transcripts of *LHCBM2* and *LHCBM8*, but the affinity towards *LHCBM6* mRNA was more than tenfold higher (Wobbe, 2007). This indicates that the effect of a direct NAB1 mediated translation repression of other LHCII isoforms is probably low.

The presence of LHCBM6 might therefore be crucial for antenna assembly, allowing an indirect control of overall PSII antenna size through regulating one isoform only. This hypothesis is supported by studies on the remodeling of PSII during state transitions. Under state II conditions, the phosphorylation of LHCII type I, including LHCBM6, triggers the dissociation of PSII megacomplexes into supercomplexes, and subsequently trimers detach from the core (Iwai et al., 2008; Drop et al., 2014b). Interestingly, phosphorylated LHCII type I partly remain at PSII (Drop et al., 2014b). Upon prolonged high PSII excitation pressure, NAB1 mediates the down-regulation of LHCBM6 abundance (4.2). Simultaneously state transitions relax, but association of returning LHCII might be lower because of reduced LHCBM6 levels. In the following, the pool

size of these unbound, 'extra' trimers (Drop et al., 2014a) could be reduced by dilution in the course of cell division (Durnford et al., 2003), as degradation of LHCII was hitherto not observed in unicellular green algae (Fujita et al., 1989; Webb and Melis, 1995). This scenario evidently requires feedback control on overall LHCII expression, and indeed transcription of most LHCII is co-regulated (Teramoto et al., 2002; Elrad and Grossman, 2004). Notably, the abundance of LHCBM6 at PSII is rather low compared to other type I isoforms (Drop et al., 2014a), presumably facilitating a relatively quick and strong regulation of protein levels. Against this background, the isoform specific control of LHCBM6 translation emerges as an efficient and fast means to regulate the attachment of LHCII to PSII before slower transcriptional responses (Dietz, 2015) come into account.

The considerations on the importance of LHCBM6 on PSII antenna assembly appear reasonable but lack isoform specific experimental data. The investigation of the distinct functions of individual LHCII is therefore a major task in future investigations (7). To this end, deregulating isoforms *in vivo* is a potent means. The knock out or knock down of LHCBM1, LHCBM2/7 and LHCBM9 (Elrad et al., 2002; Ferrante et al., 2012; Grewe et al., 2014) already revealed the respective roles of these subunits in photo-acclimation and stress responses (1.2.2). The effects of down-regulation and over-expression of the other isoforms, in the context of this work particularly of LHCBM6, on PSII structure and acclimation behavior will therefore provide fundamental insights. De-regulating protein abundances is however challenging, on the one hand because of off-target effects in RNA interference approaches and on the other hand because of cellular responses that counterbalance the induced impairment, so that direct and indirect effects are difficult to resolve (Ferrante et al., 2012; Pietrzykowska et al., 2014).

Furthermore, localization of isoforms within the thylakoid membrane provides new information. The three types of trimers at PSII (S, M and N) and those found in the 'extra' LHCII pool (Drop et al., 2014a) likely account for different light-harvesting and protective functions (Betterle et al., 2009). A combination of membrane solubilization and density centrifugation with immunodetection could be applied to this end, similar as has been performed for LHCBM9 (Grewe et al., 2014). However, specific antibodies exist only for few a isoforms. Tagging can be used as alternative approach, and *C. reinhardtii* strains expressing human influenza hemagglutinin (HA)-tagged LHCBM6 are already available (Mussgnug et al., 2005).

Experiments *in vitro* complement physiological LHCBM analyses, and the reconstitution of LHCII (Paulsen et al., 1990) as well as their isolation from thylakoids (Peter and Thornber, 1991) is well established. Very recently, LHCBM1, LHCBM2/7, LHCBM5, LHCBM6 and LHCBM9 were reconstituted and characterized regarding pigmentation and spectroscopic features (Natali

and Croce, 2015). Though differences were small and difficult to interpret, some characteristics of LHCBM6 can be outlined. Reconstituted LHCBM6 monomers had a comparatively low chlorophyll *a* to *b* ratio of 1.14, but high neoxanthin and/or loroxanthin content, and therewith resembled rather the pigmentation of the isoform involved in qE, LHCBM1, than the subunits clearly shown to impact state transitions, LHCBM5 and LHCBM2/7. Also the average fluorescence lifetime was very long and similar to LHCBM1, but the kinetic details of the fluorescence decay differed. Furthermore temperature dependent denaturation was tested, and LHCBM6 monomers appeared to be slightly more stable than other isoforms. However, the meaning of the data obtained is not clear and remains to be elucidated (Natali and Croce, 2015). Overall, only minor differences between isoforms were observed in that study, indicating that the assigned functions such as the involvement of LHCBM1 in qE (Elrad et al., 2002; Ferrante et al., 2012) are less due to intrinsic properties of the respective complex alone but rather result from interaction with other factors such as LHCSR3 in the case of energy dependent quenching (1.2.2; 6.3.1).

In future investigations, the different light-harvesting antenna isoforms could be analyzed regarding their localization at PSII (see above) as well as concerning their ability to form homo- and heterotrimers and to interact with other components of the thylakoid membrane such as the photosystems and the qE related LHCSR3 (7). The combination of such studies could provide hints on the importance of the subunits for PSII antenna stability and photoprotection, and therewith contribute to understand the role of the LHCBM6 specific NAB1 mediated translation repression.

6.3.3 Functional and temporal interrelation of photosynthetic acclimation responses

The regulation of photosynthetic electron transport and light-harvesting occurs on multiple levels and time scales, and short- to long-term responses are coordinated within the cell (1.2; 1.3). In this work, it could be shown that the interplay of state transitions on short and LHCBM translation control on longer time scales efficiently adjust photosystem II excitation pressure upon carbon dioxide limitation (4.2). In addition to the observed temporal course of light-harvesting antenna adjustments, the distinct mechanisms appear functionally and regulatory dependent, as on the one hand the accumulation of the LHCBM translation repressor is impaired in the state transition mutant *stt7* (Figure 4-6), and on the other hand the *NAB1* knock out mutant performs only limited state transitions (Figure 4-8).

Within the first detailed description of the *NAB1* knock out mutant it was reasoned that the impaired ability to perform state transitions is an indirect effect of super-stacked grana due to increased LHCII accumulation (Mussgnug et al., 2005). LHCII were supposed to stay within the

grana thylakoids, bound to PSII, implying that the mutant is locked in state I because of steric hindrances.

The 77 K fluorescence emission spectra obtained within this work (Figure 4-8), however, suggest that the *NAB1* knock out mutant is in general fully capable of performing state transitions, as carbon dioxide limitation increases PSI fluorescence, and the addition of the PET inhibitor DCMU, triggering state I in wild-type cells, also significantly decreases PSI fluorescence in the mutant. The low amplitude of state transitions under physiological conditions (Kruse et al., 1999; Mussgnug et al., 2005; Figure 4-8) might therefore be due to altered cellular energetics rather than grana stacking. The *NAB1* knock out mutant is not able to sufficiently decrease PSII antenna size under low inorganic carbon supply with the consequence of a lowered effective quantum yield (Mussgnug et al., 2005; Figure 4-3) and a high PSII excitation pressure measured as 1-qP (Figure 4-3). Enhanced PSII excitation reflects a reduced PQ pool, and increased binding of plastoquinole to cytochrome b_6f complexes triggers state II (Zito et al., 1999). This might explain the state transition phenotype of the *NAB1* knock out mutant and indicates that over-reduction of the PET chain triggers a permanent state II rather than state I in this strain under physiological conditions.

Interestingly, the *NAB1* knock out mutant was furthermore unable to increase cyclic electron flow upon carbon dioxide limitation in acetate containing media, but able to in minimal media (Figure 4-8). CO_2 limitation leads to the accumulation of reducing equivalents and increases the demand for ATP (1.1.2). Under these conditions, increased cyclic electron flow around photosystem I both regenerates electron acceptors and enhances photophosphorylation to balance the required ratio of NADPH to ATP (Lucker and Kramer, 2013; 1.1.1). Wild-type cells indeed showed elevated cyclic electron flow under low CO_2, most prominent when acetate was available, but also under photoautotrophic growth conditions (Figure 4-8). The inability of the *NAB1* knock out mutant to enhance cyclic electron flow during photoheterotrophy is consistent with the importance of NAB1 mediated translation control under this condition (Figure 4-3). The accumulation of NAB1 was much stronger under carbon dioxide limitation in acetate containing media than in minimal media (Figure 4-2). Thus, the difference between knock out mutant and wild-type strain can be expected to be larger when organic carbon is supplied simultaneously. The lower extent of cyclic electron flow could further explain the impaired growth of the NAB1-less strain under photoheterotrophy observed in this (Figure 4-4) and a previous study (Mussgnug et al., 2005). Acetate assimilation consumes ATP (1.1.2), and premising that the mutant generates less ATP because of a lower extent of cyclic electron flow, acetate is probably consumed more slowly in this strain. Indeed, acetate in the growth medium decreased faster in wild-type than *NAB1* knock out cell cultures (Figure S2).

Also the state transition mutant *stt7*, which is deficient in the state transition kinase STT7 (Fleischmann et al., 1999; Depège et al., 2003), showed an impaired acclimation to fluctuating carbon supply (4). Hitherto, evidences on alterations in growth or long-term photosynthetic regulation, as observed for the respective knock out mutants in higher plants (Bonardi et al., 2005; Pesaresi et al., 2009; Tikkanen and Aro, 2014; 1.3.1), are rare for the algal strain. The STT7 knock out mutant grows under photoautotrophic and photoheterotrophic conditions, both with high or low carbon dioxide supply (Fleischmann et al., 1999; Depège et al., 2003; Cardol et al., 2009). Cardol et al. (2009) revealed a growth phenotype by inhibiting mitochondrial electron transport, indicating that respiratory ATP generation counterbalances the state transition deficiency. Furthermore, *stt7* was shown to be affected by high light intensities more than wild-type cells (Allorent et al., 2013). Interestingly, the reduced ability of the analog *A. thaliana* mutant *stn7* to cope with fluctuating light applies only to young, developing seeds (Tikkanen and Aro, 2014). Mature leaves apparently compensated the deficiency, similar to *C. reinhardtii* cells.

In the work presented here, NAB1 expression was deregulated in *stt7* upon a switch from inorganic to organic carbon supply (Figure 4-6; 6.2.1). Whether the altered NAB1 accumulation is directly due to the lack of STT7 or an indirect effect of other disturbed signaling pathways (Figure 6-1, dashed arrows) needs to be determined (7). Interestingly, a small, but significant growth perturbation under fluctuating carbon dioxide was observed for *stt7* (Figure 4-9), disclosing an impaired acclimation under physiological conditions, without high light stress or inhibition of mitorespiration (see above). This indicates that the lack of the state transition kinase can be compensated under stable conditions in the long term, but not in a fluctuating environment.

Overall, both state transitions and LHCBM translation control emerged as essential mechanisms regulating light-harvesting at photosystem II under varying carbon supply in the work presented here. The *NAB1* knock out mutant was unable to adjust photosystem II antenna size under carbon dioxide limitation, and showed an impaired growth under these conditions (4.2). The lack of efficient translation repression was furthermore shown to cause an over-reduced electron transport chain, consistent with a permanent state II transition and the inability to increase cyclic electron flow upon carbon dioxide limitation in a photoheterotrophic growth regime (4.3.1). The state transition mutant *stt7* was impaired in accumulating NAB1 under fluctuating carbon supply and exhibited a small but significant reduction in growth rate under these conditions (4.3.1). The findings discussed in this subsection further outline the importance of a balanced photosynthetic performance for both inorganic and organic carbon metabolism and indicate a regulatory circuit of state transitions and LHCBM translation. Subsequent work could disentangle further details of the interrelations of spatially and temporally distinct light-harvesting acclimation responses (7).

6.3.4 PSII antenna size adjustment in response to light and carbon supply

Carbon dioxide limitation induced a reduction of photosystem II antenna size via NAB1 mediated LHCBM translation control in the work presented here (4.2). Interestingly, although regarded as important acclimation strategy, only few studies point towards biochemical or functional PSII antenna size adjustments in *C. reinhardtii* under physiological conditions, as outlined below.

Optimal light to biomass conversion is a main determinant for sustainable algae mass culture, and a small but efficient light-harvesting antenna is thought to increase light capture and penetration (Polle et al., 2002; Melis, 2009). Several attempts were therefore undertaken to create algae with truncated antennae, including down-regulating the synthesis of LHCII specific pigments (Polle et al., 2001; Perrine et al., 2012), knock down of LHCII (Mussgnug et al., 2007; Oey et al., 2013), impairing LHCBM protein import (Kirst et al., 2012; Mitra et al., 2012; Kirst et al., 2014) and increased NAB1 mediated LHCBM translation repression (Beckmann et al., 2009).

In contrast to the engineered algal strains, there is hitherto little evidence for altered light-harvesting antenna sizes in wild-type *C. reinhardtii* under varying environmental conditions. It is reasonable that photosynthetic organisms adjust their light-harvesting apparatus to the light availability and the cellular demand for energy in order to optimize photosynthetic performance and minimize photooxidative damage (Erickson et al., 2015). Accordingly, higher plants were shown to decrease PSII antenna size for instance in high light (Morosinotto et al., 2006; Ballottari et al., 2007), with light intensities above 350 μmol photons m^{-2} s^{-1} generally referred to as high light in this thesis (Niyogi, 2009; 1.2.2). A similar response was reported for *Skeletonema costatum, Dunaliella salina* and *Dunaliella viridis*, while other microalgae such as *Dunaliella tertiolecta* did not reduce PSII antenna size under increased light intensities (Falkowski and Owens, 1980; Smith et al., 1990; Melis et al., 1998; Gordillo et al., 2001), indicating that acclimation strategies are species specific (Bonente et al., 2012).

In *C. reinhardtii*, the amount of LHCBM proteins clearly decreases upon increased light intensities due to transcriptional and post-transcriptional regulations (Teramoto et al., 2002; Durnford et al., 2003; McKim and Durnford, 2006; Bonente et al., 2012; 5; Figure 6-2, 6-3), while expression of other LHC-like proteins with protective functions such as LHCSR3 is induced (Teramoto et al., 2004; Peers et al., 2009; Bonente et al., 2011; 1.2.2; 6.3.1). Concordantly, the cellular chlorophyll content declines by 50 to 60% within six hours of moderate high light (400 μmol photons m^{-2} s^{-1}; Shapira et al., 1997; Bonente et al., 2012).

However, a lowered LHCII and chlorophyll level under high light does not necessarily reflect an altered PSII antenna size; that is the actual amount of pigments efficiently transferring excitation

energy to a photosystem II core. The ratio of light-harvesting proteins to core subunits can be approached directly by immunoblot detection or indirectly by measuring the chlorophyll *a* to *b* ratio, as the latter pigment is exclusively bound to the outer antenna. The functional antenna size of PSII can be directly determined through the kinetics of chlorophyll *a* fluorescence induction. All these methods were combined to study the acclimation of the photosynthetic apparatus of cells grown under low and high light (Bonente et al., 2012). In that study, the chlorophyll *a* to *b* ratio ranged between 2.21 in low and 2.30 in high light acclimated cells, and a small, gradual decrease in functional PSII antenna size was observed, but the differences were not statistically significant. The authors therefore proposed that the PSII antenna size is not changing substantially under the light intensities tested, and that the overall decrease in cellular chlorophyll content is due to a reduction in photosystem number. On the other hand, applying similar light intensities, Neale and Melis (1986) reported that the amount of chlorophylls per PSII ranged from 620 in low to 460 in high light grown cells, accounting for a decline by 25%.

In this work, a clear reduction of photosystem II antenna size upon carbon dioxide limitation in acetate containing media could be shown by combining biochemical and biophysical methods (4.2). The ratio of LHCII to PSII core subunit proteins was lower and the functional PSII antenna size was decreased by 50% after twelve hours of carbon dioxide limitation in acetate containing media (Figure 4-3). An overall reduced fluorescence yield was observed in air adapted cells under photoautotrophic conditions before, but this was mainly attributed to a state II transition, although Spalding et al. (1984) also observed a slightly lower half-rise time of fluorescence induction (Spalding et al., 1984; Palmqvist et al., 1990; Falk and Palmqvist, 1992). In the study presented here, state transitions reversed after two to four hours (Figure 4-6), similar to observations on photoautotrophically grown cells (Iwai et al., 2007), indicating that expression based acclimation responses replace short-term responses on longer timer scales. Taking the reports on an unaltered or only slightly changed PSII antenna size under varying incident light intensities into account (Neale and Melis, 1986; Bonente et al., 2012; see above), this study (4.2) points towards carbon sources as main determinants of PSII antenna size in *C. reinhardtii*.

6.4 Regulation of light-harvesting in *C. reinhardtii* – A model

Light-harvesting is strictly regulated on multiple levels in several cellular compartments of *C. reinhardtii* over time (1.2). This complex control underlines the importance of balanced light energy input for the algal cell. The mechanisms adjusting light-harvesting at photosystem II depending on light and carbon availability described in the studies presented here (4.2; 5) integrate into a model depicting regulatory responses that occur under defined PSII excitation pressure states (Figure 6-3). Evidently, the conditions outlined in the following represent snapshots which in reality merge, creating intermediate situations (Brautigam et al., 2010).

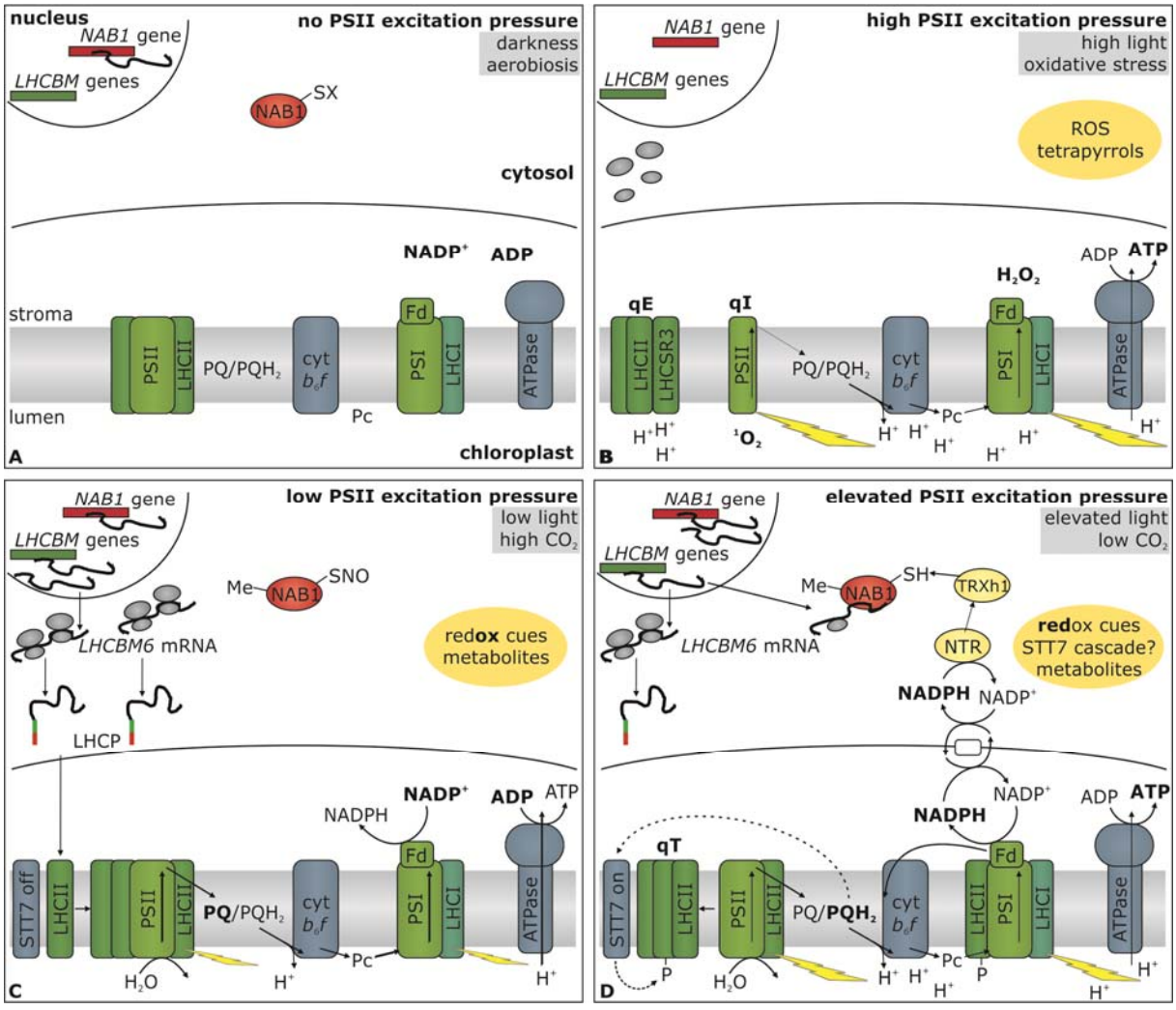

Figure 6-3 Regulation of light-harvesting dependent on the prevailing PSII excitation pressure.
A model depicting four cellular states under environments (grey boxes) causing no (A), low (C), elevated (D), and high (B) PSII excitation pressure. Chloroplastic and retrograde communication (yellow ovals) adjust short- and long-term responses eventually regulating light-harvesting at PSII. Signaling pathways controlling LHCBM translation control are depicted in detail in Figure 6-1. The physiology behind the states outlined is explained in the text (6.4). Abbreviations as described before (1; Figures 6-1 and 6-2).

In darkness (Figure 6-3A), energy is mainly acquired via starch degradation (Johnson and Alric, 2013; 1.1.2). Photosystem II is not excited, hence no quenching processes occur. LHCII transcript abundance is low (Teramoto et al., 2002); the translation repressor NAB1 is not methylated and is therefore inactive (Blifernez et al., 2011).

When energy input at photosystem II is limiting, for instance under low light and/or excess carbon dioxide, PSII excitation pressure is low and intersystem as well as soluble electron carriers are in an oxidized state (Figure 6-3C). The state transition kinase STT7 is inactive because of an oxidized PQ pool (Horton and Black, 1980; Zito et al., 1999), and LHCII are associated with PSII (state I). Retrograde signals communicate the lack of energy input to the other cellular compartments (Figure 6-3, yellow oval). In the nucleus, transcription of LHCII is increased (Teramoto et al., 2002; Humby et al., 2009). Methylation of arginines renders NAB1 in a generally active state (Blifernez et al., 2011), but RNA binding activity is low due to nitrosylation of cysteine 226 (5). Furthermore, NAB1 is expressed at low rates (4). The high *LHCBM* transcription together with relaxed translation control allows efficient light-harvesting protein synthesis under energy limited conditions.

An increase in light intensity or a lack of downstream electron acceptors under carbon dioxide limitation causes an over-reduction of the photosynthetic electron transport chain and elevated PSII excitation pressure (Figure 6-3D). In order to decrease pressure on photosystem II, dissipative pathways are induced. A reduced plastoquinone pool activates STT7 (Zito et al., 1999), and a state II transition (qT) efficiently reduces PSII absorption cross section in the short term (Iwai et al., 2007; 4). Retrograde signals communicate the excess energy input to the other cellular compartments. LHCII transcription is decreased in the nucleus (Teramoto et al., 2002; Humby et al., 2009), and LHCBM protein synthesis in the cytosol is repressed, eventually decreasing PSII excitation pressure in the long term (Mussgnug et al., 2005; 4; 5).

Both elevated light intensity and carbon dioxide limitation increase PSII excitation pressure (Figure 6-3D), but the signaling pathways that lead to NAB1 mediated LHCBM translation repression are distinct or diverge (6.2; Figure 6-1). Carbon dioxide limitation triggers the promoter based increase of NAB1 expression, most prominently when an organic carbon source is available as well (4). In contrast, elevated light is sensed via redox based post-translational NAB1 activity regulation (Wobbe et al., 2009; 5), and most likely the cytosolic system of thioredoxin h1 (TRX h1) and NADPH dependent thioredoxin reductase (NTR) uses excess reducing power to denitrosylate and therewith fully activate NAB1 (5). Furthermore, while carbon dioxide limitation and acetate supply clearly lead to a smaller PSII antenna size (4), the decreased LHCII levels under elevated light (5) are apparently accompanied by a general

reduction of photosystem number rather than antenna size alterations (6.3.4). Instead, a low number of photosystems together with enhanced regeneration of electron acceptors through acceleration of the Calvin cycle (Dietz, 2015) could relieve excitation pressure on PSII under elevated light.

A situation causing a perilously high PSII excitation (Figure 6-3B), for instance upon sudden high light, is characterized by the generation of reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2), and increased damage of the PSII core protein D1 (qI) (Niyogi, 2009; Dietz, 2015). LHCII dissociate from PSII, and expression and protonation of LHCSR3 fosters the dissipation of excess energy as heat (qE) (Peers et al., 2009; Bonente et al., 2011; Tokutsu and Minagawa, 2013; 1.2.2). ROS and tetrapyrrole signals are involved in transient repression of LHCII transcription (Teramoto et al., 2002; Durnford et al., 2003; Elrad and Grossman, 2004; Formighieri et al., 2012; Suzuki et al., 2012). LHCBM protein synthesis is low because of a ROS triggered global polysome disassembly (Durnford et al., 2003; McKim and Durnford, 2006; 1.2.3; Figure 6-2A), and also NAB1 expression is strongly decreased (4).

The model describes the regulation of light-harvesting in the green alga *C. reinhardtii* under aerobic, nutrient replete environments; conditions under which light and carbon availability largely determine photosynthetic performance (1.1; 4; 5; 6.3.4). The presence of oxygen responsive elements on the *NAB1* promoter (4.3.2; 6.2.1) already indicates a putative role of LHCBM translation repression under anaerobic, fermentative conditions. Also nutrient deprivations dramatically impact photosynthesis, both directly because of limited regeneration of photosynthetic proteins and pigments, and indirectly because of metabolic changes that alter the demand for energy equivalents (Grewe et al., 2014; Schmollinger et al., 2014). Whether NAB1 mediated LHCBM translation repression is involved in the photosynthetic remodeling under nutrient deprivation remains to be elucidated (7).

Control of light-harvesting protein synthesis is an essential mechanism of acclimation in evolutionarily diverse photosynthetic organisms, from cyanobacteria to vascular plants (Frigerio et al., 2007; Wobbe et al., 2008; Floris et al., 2013; Gutu et al., 2013). However, NAB1 like proteins are until now found only in the close *C. reinhardtii* relatives *C. incerta* and *Volvox carteri* (Nematollahi et al., 2006; Popescu et al., 2006), and the combination of cold shock domain and RNA recognition motif appears to be unique for this protein (1.2.3). It will be an interesting for future research to unravel the evolution of NAB1 mediated LHCBM translation repression in these green algae. However, control of LHCBM protein synthesis is apparently a fundamental response of photosynthetic organisms to efficiently regulate light-harvesting (see above), suggesting that similar mechanisms as those investigated and discussed here probably exist in

other algae and plants. Unraveling the factors and underlying regulatory networks could be an intriguing topic for subsequent research (7).

In summary, the studies presented here depict regulatory circuits of short- and long-term acclimation responses occurring in the nucleus, cytosol and chloroplast in *C. reinhardtii*. LHCBM translation control was disclosed as central regulatory hub adjusting the amount of light-harvesting proteins to the demand of the photosynthetic apparatus upon changes in PSII excitation pressure under physiological conditions (Figure 6-3C,D). Light and carbon dioxide availability emerged as major factors influencing light-harvesting protein synthesis, however, on distinct signaling pathways that control the abundance and activity of NAB1 (Figure 6-1). Evidently, a multi-level regulatory network adjusts photosynthetic light capture to optimize photon conversion efficiencies.

7 Conclusions and perspectives

Light and carbon availability largely determined light-harvesting at photosystem II in *Chlamydomonas reinhardtii* in the work presented here, with NAB1 mediated LHCBM translation repression as key lement of photosynthetic acclimation responses. The studies contained within this thesis revealed a regulatory link of short-term protective responses in the chloroplast and nuclear as well as cytosolic expression control under fluctuating carbon supply, and disclosed the dynamic, redox based regulation of light-harvesting protein synthesis under varying incident light intensities.

In particular, the questions raised (2) could be answered as follows.

Under which environmental and physiological conditions does NAB1 mediated translation control adjust light-harvesting antenna sizes?

Conditions that reduce chloroplastic intersystem and soluble electron carriers increase NAB1 mediated LHCBM translation repression. In the work presented here, this was triggered by carbon dioxide limitation and acetate supply as well as elevated light, but not high light. Carbon dioxide limitation in a heterotrophic growth regime caused a reduction in functional PSII antenna size by more than 50%.

Does NAB1 expression regulation influence LHCII translation control in addition to post-translational activity switches?

Yes, an increased NAB1 expression is crucial for PSII antenna size reduction under carbon dioxide limitation. In contrast to wild-type algae, a strain driving *NAB1* transcription from a different promoter was impaired in growth under fluctuating carbon supply. This indicates that post-translational modifications are not sufficient for the adjustment of LHCBM protein synthesis under this condition, and that *NAB1* promoter regulation is essential.

Which molecular mechanisms and signaling pathways underlie the redox based NAB1 activity control?

The cysteine residue at amino acid position 226 is located in the RNA recognition motif of NAB1 and is crucial for the redox control of the protein's RNA binding activity. Under low light, nitrosylation of this residue decreases NAB1 activity. Denitrosylation occurs under elevated light, and in this the thiol state, the protein actively binds *LHCBM6* mRNA and represses protein synthesis. Cysteine reduction is probably mediated by a system of cytosolic thioredoxin h1 and thioredoxin reductase which uses reducing equivalents in the form of NADPH exported from the chloroplast.

How do short- and long-term regulatory responses interrelate to adjust PSII light-harvesting capacity?
LHCBM translation repression replaces state transitions as initial PSII protective mechanism on longer time scales. Besides this temporal course, the regulation of short- and long-term responses appears to be intertwined. NAB1 accumulation was impaired in the state transitions mutant *stt7* and the *NAB1* knock out mutant was in a permanent state II under the growth conditions examined, indicating an interdependence of spatially and temporally distinct regulatory hubs.

How do chloroplast, cytosol, and nucleus communicate to coordinate light-harvesting regulation?
Cellular compartments communicate via retrograde signals towards and anterograde signals emerging from the nucleus. Signals from the chloroplast requiring photosynthetic electron transport and/or the state transition kinase STT7 control nuclear *NAB1* transcription and therewith PSII antenna adjustment under fluctuating carbon supply. A 152 bp element on the nuclear *NAB1* promoter is sufficient for the sensing and implementation of the signal(s) and contains hitherto unknown *cis*-regulatory elements. High light intensities that cause oxidative stress decrease NAB1 levels, and LHCBM protein abundance is likely reduced due to a ROS triggered global polysome disassembly together with a transient decline of *LHCBM* transcript levels. Nitrosative signals increase LHCBM translation by decreasing NAB1 activity in the cytosol under low light. Upon elevated light, a reduction of nitrosylated NAB1 cysteine 226 enhances translation repression and causes decreased LHCBM levels. Probably, accumulating stromal reducing power is released into the cytosol via transport systems such as the malate oxaloacetate shuttle. Overall, communication involves redox cues such as nitric oxide, NADPH and oxidative species, and the thylakoid kinase STT7 is important for signaling under reducing conditions.

To summarize, the work presented here shows that control of light-harvesting protein synthesis and coordination of short- and long-term responses are essential for acclimation of *C. reinhardtii* to fluctuating carbon and light supply. The findings presented and discussed in this thesis raise further questions to be addressed in the future, which are outlined below.

The regulation of LHCBM protein synthesis via NAB1 emerged as a central regulatory hub for the photosynthetic acclimation under varying light and carbon supply, and coordination of acclimation responses was shown to involve chloroplast to cytosol and to chloroplast to nucleus signaling (6.2). A 152 bp element of the *NAB1* promoter contains essential, hitherto unknown regulatory elements, and the fast increase of *NAB1* transcript levels under carbon dioxide limitation indicates that preexisting signaling pathways and transcription factors are used (4; 6.2.1). This knowledge can be applied in future work to identify transcription factors involved by using the 152 bp *NAB1* promoter sequence as a DNA probe for affinity purification (Brune et al., 2011). The reporter system developed (3; 4; 6.1) can be employed as a tool for signaling

studies applying inhibitors (Trebst, 2007) or reagents that mimic or induce known signaling cues (Fischer et al., 2005; Voß et al., 2011), and to determine *NAB1* promoter activity under environments that trigger over-reduction of the photosynthetic electron transport chain, such as hypoxia or low temperatures (6.2.1). Based on the reporter strains generated in this work, knock out cell lines could be created by random DNA insertion and screened for the inability of carbon dioxide responsive luciferase expression. Similarly, introduction of the reporter construct into *stt7* and subsequent mutagenesis allows searching for suppressor mutations regenerating wild-type NAB1 expression. Such forward genetic approaches thus enable the identification of upstream regulatory factors. Overall, the carbon dioxide responsive 152 bp *NAB1* promoter fragment and the perturbed signaling in *stt7* as shown in the present thesis (4) provide powerful tools to elucidate the communication pathways and novel regulatory promoter elements involved in the control of photosynthesis associated genes encoded in the nucleus by retrograde signals.

The mechanism of redox based LHCBM translation control revealed in this work (5; 6.2.2) provides the basis for future studies analyzing chloroplast to cytosol signaling. Inhibition of malate dehydrogenase by fluoromalate (Berry and Kun, 1972) could be applied to investigate the importance of the translocation of reducing equivalents by the malate oxaloacetate shuttle, and the role of thioredoxin h1 for NAB1 reduction under elevated light could be determined *in vivo* by analyzing the respective *C. reinhardtii* knock out mutant (Sarkar et al., 2005). The extent of NAB1 nitrosylation could be analyzed in a strain containing nitrate reductase and by application of a nitric oxide synthase inhibitor (Moreau et al., 2010) to investigate the source of nitric oxide. Moreover, though *NAB1* promoter control is clearly important for LHCBM translation regulation under fluctuating carbon supply (4.1), the activity of the repressor could be regulated additionally under this condition. High turnover of the Calvin cycle leads to the consumption of reducing power. This decreases the export of reducing equivalents into the cytosol and presumably slows down thioredoxin mediated NAB1 reduction. The level of NAB1 nitrosylation could therefore increase under this condition, similar to observations under low light (5). Unraveling the interplay of the different levels of NAB1 regulation, including promoter control, arginine methylation and cysteine nitrosylation, will provide new insights into the connection of cytosolic and nuclear control of light-harvesting protein synthesis.

The translation repressor NAB1 shows a strong preference towards the LHCII isoform LHCBM6 (Mussgnug et al., 2005; Wobbe, 2007), nevertheless the protein abundance of different types of LHCII types appeared co-regulated in this (4) and previous studies (Wobbe et al., 2009; Blifernez et al., 2011). In this work, the hypothesis was raised that LHCBM6 might be important for PSII antenna assembly (6.3.2). In order to test this assumption, this isoform should be characterized *in vitro* and *in vivo*. As previous analyses of reconstituted and isolated LHCII

isoforms revealed only little differences in between isoforms (Natali and Croce, 2015), future studies should emphasize the trimerization behavior, the characterization of homo- and heterotrimers and the interaction with components of thylakoid membrane, such as photosystem I and II as well as LHCSR3. The localization of the isoforms *in vivo* could reveal new information (Grewe et al., 2014) as the trimers at PSII termed S, M and N as well as those in the extra pool are probably implicated in different regulatory responses (Betterle et al., 2009; Drop et al., 2014a). The algal strains expressing human influenza hemagglutinin (HA)-tagged LHCBM6 (Mussgnug et al., 2005; 5.2) could be useful for such localization studies. Furthermore, strains with reduced or increased LHCBM6 level via knock down and over-expression could provide new insights into the importance of this isoform for photosynthetic acclimation as well as for light-harvesting and non-photochemical quenching. As off-target and compensator effects complicate such studies (Ferrante et al., 2012; Pietrzykowska et al., 2014), a combination of *in vitro* and *in vivo* studies provides the most reliable findings.

The work presented here highlights novel aspects of the regulatory circuit of short- and long-term mechanisms controlling light-harvesting capacities at photosystem II (4; 6.3.3). Light and carbon supply influence the demand for light-harvesting proteins and trigger distinct regulatory responses (Figures 6-1). In this work, the effect of fluctuating carbon dioxide was tested under elevated light (4) and the effect of moderate changes in incident light intensity was tested under low carbon dioxide supply (5; Figure 6-2B). Future work could complete the picture by an integrated study of light-harvesting regulation in wild-type algae, the *STT7* knock out mutant and cell lines expressing no or cysteine mutated versions of NAB1 upon moderate changes in PSII excitation pressure, triggered by low light/high CO_2, low light/low CO_2, elevated light/high CO_2, and elevated light/low CO_2. The different levels of NAB1 regulation (6.2) and *LHCBM* transcription control as well as short-term responses should be taken into account. Similar to studies investigating the ROS network in which the effects of light and oxygen on the generation of oxidative stress responses were disentangled (Barth et al., 2014), the signaling pathways and responses involved in the acclimation to carbon supply and moderate changes in light intensity could be unraveled. Furthermore, the extent of energy dependent quenching should be investigated as evidence for an interrelation of qE and qT is growing (6.3.1). The involvement of high energy quenching under the conditions chosen could be studied by immunoblot detection of LHCSR3 levels on the one hand and kinetic analysis of fluorescence induction and NPQ relaxation on the other hand (Iwai et al., 2007).

Adjustment of light-harvesting protein synthesis is evidently a key element of photosynthetic acclimation in *C. reinhardtii* (6.3.4). It is reasonable that LHCII translation control is a fundamental instrument in photosynthetic organisms, suggesting that mechanisms similar to

those investigated in this work exist in other algae and plants. Indeed, there is evidence for post-transcriptional light-harvesting regulation in model organisms of diverse evolutionary lineages (Frigerio et al., 2007; Wobbe et al., 2008; Floris et al., 2013; Gutu et al., 2013). As database research reveals that NAB1 like proteins, combining a cold shock domain with a RNA recognition motif, are apparently not found apart from close *C. reinhardtii* relatives (Mussgnug et al, 2005; 1.2.3; 6.4), other RNA binding proteins with a different domain combination probably fulfill its function. Such proteins could be identified in a biochemical approach using LHCII transcripts as a probe on an affinity column (Danon and Mayfield, 1994). The identification of LHCII translation regulators in other organisms would be highly interesting in order to understand the diversity of photosynthetic acclimation strategies and because this knowledge can be applied, as successfully shown for *C. reinhardtii* (6.3.4), to optimize light capture and penetration in mass cultures of algae with a greater biotechnological relevance.

In summary, a sophisticated regulatory network orchestrates the multi-compartmental regulation of light-harvesting in *C. reinhardtii*. A major challenge for future work is to further disentangle the interrelation of spatially and temporally distinct light-harvesting regulation responses in this and other photosynthetic organisms. The work presented in this thesis provides novel insights into the underlying intracellular communication and useful tools to analyze the concerting signaling pathways that balance photosynthetic light capture to ensure optimal photosynthetic performance.

Appendix

Figures S1 to S4 are part of publication II supplemental information (Berger et al. 2014).

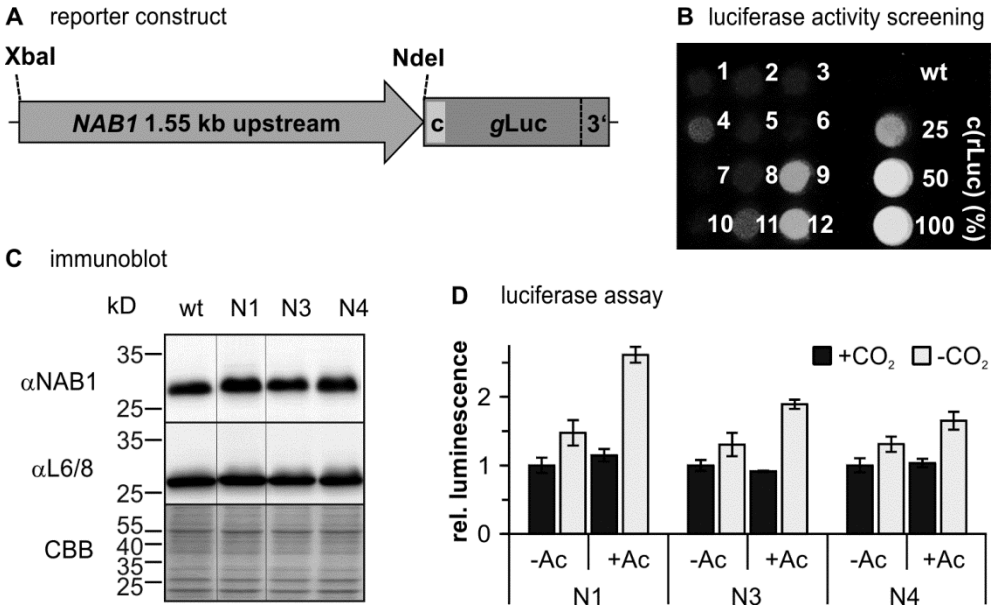

Figure S1 Promoter::reporter construct design, screening and characterization of cell lines expression *NAB1*::*g*Luc reporter.
(A) A 1.55 kb element upstream the NAB1 translation start was fused to *Gaussia* luciferase gene (*g*Luc) containing a native *Chlamydomonas* secretion signal (c). (B) Detection of luciferase activity in culture supernatants of twelve cell lines chosen by plate based screening (10). Recombinant luciferase (rLuc) served as standard. 4, 9, and 12 were chosen and renamed N1, N3 and N4. (C) Validation of NAB1 (αNAB1) and LHCBM6/8 (αL6/8) expression in chosen cell lines by immunoblotting. Coomassie briliant blue (CBB) staining serving as loading control. (D) Luciferase assay as described in Figure 4-2B. Data represent means and SD of individual measurements (n=3).

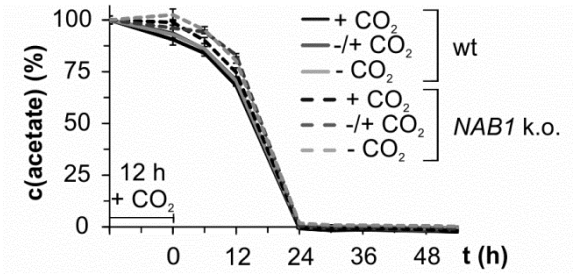

Figure S2 Acetate consumption during photoheterotrophic and mixotrophic growth.
Cells of the wt (solid lines) and *NAB1* k.o. strain (dashed lines) were cultivated as in Figures 4-3 and 4-4 under continuous CO_2 supply (black lines), air bubbling (light grey) or under fluctuation CO_2 levels in 6 h intervals (dark grey). Remaining concentration of acetate in the culture media was determined relative to TAP media. Representative triplicate determination of one out of three biological replicates (n=3).

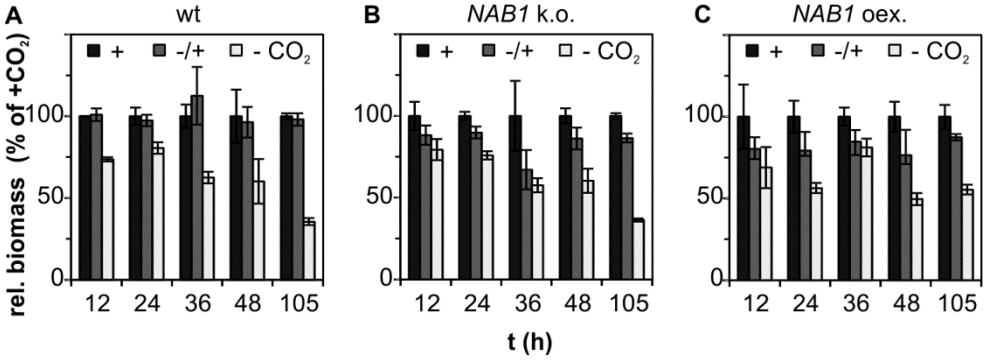

Figure S3 Biomass accumulation of wt (A), *NAB1* k.o. (B) and *NAB1* oex (C) during growth.
Cultures were grown as described in Figures 4-3 to 4-5. Growth was determined as dry biomass. Data represent relative values of absolute numbers shown in Figures 4-4 and 4-5 with condition +CO_2 set to 100% for each strain and time.

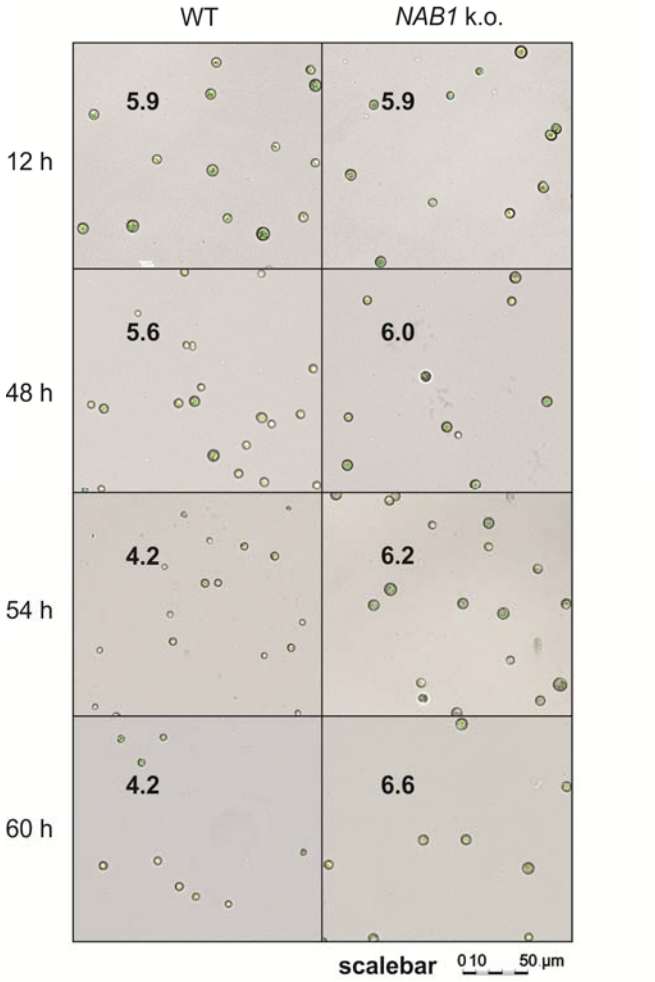

Figure S4 Cell appearance during mixotrophic growth.
Representative light microscopic images of wt (left panels) and *NAB1* k.o. strain (right panels), cultured in acetate containing media and 3 % (v/v) carbon dioxide as described in Figures 4-3 and 4-4. Values in each panel represent maximum of size distributuion (µm) determined with cell counter.

Figure S5 Annotated *NAB1* promoter sequence

Candidate *cis*-regulatory elements in the 1548 bp element upstream of *NAB1* translation start as described in Table 4-2 and Figure 6-1 are marked.

orange: elements involved in copper and hypoxia signaling (curecore; Quinn et al., 2002; Kropat et al., 2005)
yellow: motifs conferring CO_2-responsiveness (Winck et al., 2013a)
blue: low temperature response elements (ltre; Jiang et al., 1996; Dunn et al., 1998; Kim et al., 2002)
green: *experimentally determined transcription start site; TATA-box and AT-rich region are putative alternative start sites
grey: 5'UTR

References

Akter, S., Huang, J., Waszczak, C., Jacques, S., Gevaert, K., Van Breusegem, F., and Messens, J. (2015). Cysteines under ROS attack in plants: a proteomics view. *J. Exp. Bot.* doi 10.1093/jxb/erv1044.

Alergand, T., Peled-Zehavi, H., Katz, Y., and Danon, A. (2006). The chloroplast protein disulfide isomerase RB60 reacts with a regulatory disulfide of the RNA-binding protein RB47. *Plant Cell Physiol.* **47**:540-548.

Allen, J.F. (1992). Protein phosphorylation in regulation of photosynthesis. *BBA-Bioenergetics* **1098**:275-335.

Allmer, J., Naumann, B., Markert, C., Zhang, M., and Hippler, M. (2006). Mass spectrometric genomic data mining: Novel insights into bioenergetic pathways in *Chlamydomonas reinhardtii*. *Proteomics* **6**:6207-6220.

Allorent, G., Tokutsu, R., Roach, T., Peers, G., Cardol, P., Girard-Bascou, J., Seigneurin-Berny, D., Petroutsos, D., Kuntz, M., Breyton, C., et al. (2013). A dual strategy to cope with high light in *Chlamydomonas reinhardtii*. *Plant Cell* **25**:545-557.

Alric, J. (2014). Redox and ATP control of photosynthetic cyclic electron flow in *Chlamydomonas reinhardtii*: (II) Involvement of the PGR5–PGRL1 pathway under anaerobic conditions. *BBA-Bioenergetics* **1837**:825-834.

Alric, J., Lavergne, J., and Rappaport, F. (2010). Redox and ATP control of photosynthetic cyclic electron flow in *Chlamydomonas reinhardtii* (I) aerobic conditions. *BBA-Bioenergetics* **1797**:44-51.

Anderson, J.M., Chow, W.S., and Park, Y.-I. (1995). The grand design of photosynthesis: Acclimation of the photosynthetic apparatus to environmental cues. *Photosynth. Res.* **46**:129-139.

Asada, K. (2000). The water-water cycle as alternative photon and electron sinks. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **355**:1419-1431.

Astier, J., Rasul, S., Koen, E., Manzoor, H., Besson-Bard, A., Lamotte, O., Jeandroz, S., Durner, J., Lindermayr, C., and Wendehenne, D. (2011). S-nitrosylation: an emerging post-translational protein modification in plants. *Plant Sci.* **181**:527-533.

Auerbach, C. and Robson, J.M. (1944). Production of mutations by allyl isothiocyanate. *Nature* **154**:81-81.

Ballottari, M., Dall'Osto, L., Morosinotto, T., and Bassi, R. (2007). Contrasting behavior of higher plant photosystem I and II antenna systems during acclimation. *J. Biol. Chem.* **282**:8947-8958.

Barber, J. and Andersson, B. (1992). Too much of a good thing - light can be bad for photosynthesis. *Trends Biochem. Sci.* **17**:61-66.

Barth, J., Bergner, S.V., Jaeger, D., Niehues, A., Schulze, S., Scholz, M., and Fufezan, C. (2014). The interplay of light and oxygen in the reactive oxygen stress response of *Chlamydomonas reinhardtii* dissected by quantitative mass spectrometry. *Mol. Cell. Proteomics* **13**:969-989.

Bateman, J.M. and Purton, S. (2000). Tools for chloroplast transformation in *Chlamydomonas*: expression vectors and a new dominant selectable marker. *Mol. Gen. Genet.* **263**:404-410.

Baudouin, E. (2011). The language of nitric oxide signalling. *Plant Biol.* **13**:233-242.

Beckmann, J., Lehr, F., Finazzi, G., Hankamer, B., Posten, C., Wobbe, L., and Kruse, O. (2009). Improvement of light to biomass conversion by de-regulation of light-harvesting protein translation in *Chlamydomonas reinhardtii*. *J. Biotech.* **142**:70-77.

Bedbrook, J.R. (1980). Molecular cloning and sequencing of cDNA encoding the precursor to the small subunit of chloroplast ribulose-1, 5-bisphosphate carboxylase. *Nature* **287**:692-697.

Bedhomme, M., Adamo, M., Marchand, C.H., Couturier, J., Rouhier, N., Lemaire, S.D., Zaffagnini, M., and Trost, P. (2012). Glutathionylation of cytosolic glyceraldehyde-3-phosphate dehydrogenase from the model plant *Arabidopsis thaliana* is reversed by both glutaredoxins and thioredoxins *in vitro*. *Biochem. J.* **445**:337-347.

Bellafiore, S., Ferris, P., Naver, H., Göhre, V., and Rochaix, J.-D. (2002). Loss of Albino3 leads to the specific depletion of the light-harvesting system. *Plant Cell* **14**:2303-2314.

Benhar, M., Forrester, M.T., Hess, D.T., and Stamler, J.S. (2008). Regulated protein denitrosylation by cytosolic and mitochondrial thioredoxins. *Science* **320**:1050-1054.

Benhar, M., Forrester, M.T., and Stamler, J.S. (2009). Protein denitrosylation: enzymatic mechanisms and cellular functions. *Nat. Rev. Mol. Cell Bio.* **10**:721-732.

Benhar, M., Thompson, J.W., Moseley, M.A., and Stamler, J.S. (2010). Identification of S-nitrosylated targets of thioredoxin using a quantitative proteomic approach. *Biochemistry* **49**:6963-6969.

Bennett, J. (1977). Phosphorylation of chloroplast membrane polypeptides. *Nature* **269**:344-346.

Berger, H., Blifernez-Klassen, O., Ballottari, M., Bassi, R., Wobbe, L., and Kruse, O. (2014). Integration of carbon assimilation modes with photosynthetic light capture in the green alga *Chlamydomonas reinhardtii*. *Mol. Plant* **7**:1545-1559.

Berry, M.N. and Kun, E. (1972). Rate-limiting steps of gluconeogenesis in liver cells as determined with the aid of fluoro-dicarboxylic acids. *Eur. J. Biochem.* **27**:395-400.

Betterle, N., Ballottari, M., Zorzan, S., de Bianchi, S., Cazzaniga, S., Dall'Osto, L., Morosinotto, T., and Bassi, R. (2009). Light-induced dissociation of an antenna hetero-oligomer is needed for non-photochemical quenching induction. *J. Biol. Chem.* **284**:15255-15266.

Blifernez-Klassen, O., Klassen, V., Doebbe, A., Kersting, K., Grimm, P., Wobbe, L., and Kruse, O. (2012). Cellulose degradation and assimilation by the unicellular phototrophic eukaryote *Chlamydomonas reinhardtii*. *Nat. Commun.* **3**:Nr.1214.

Blifernez, O., Wobbe, L., Niehaus, K., and Kruse, O. (2011). Protein arginine methylation modulates light-harvesting antenna translation in *Chlamydomonas reinhardtii*. *Plant J.* **65**:119-130.

Bogen, C., Klassen, V., Wichmann, J., Russa, M.L., Doebbe, A., Grundmann, M., Uronen, P., Kruse, O., and Mussgnug, J.H. (2013). Identification of *Monoraphidium contortum* as a promising species for liquid biofuel production. *Bioresource Technol.* **133**:622-626.

Bohne, A.-V., Schwarz, C., Schottkowski, M., Lidschreiber, M., Piotrowski, M., Zerges, W., and Nickelsen, J. (2013). Reciprocal regulation of protein synthesis and carbon metabolism for thylakoid membrane biogenesis. *PLoS Biol.* **11**:e1001482.

Bonardi, V., Pesaresi, P., Becker, T., Schleiff, E., Wagner, R., Pfannschmidt, T., Jahns, P., and Leister, D. (2005). Photosystem II core phosphorylation and photosynthetic acclimation require two different protein kinases. *Nature* **437**:1179-1182.

Bonaventura, C. and Myers, J. (1969). Fluorescence and oxygen evolution from *Chlorella pyrenoidosa*. *BBA-Bioenergetics* **189**:366-383.

Bonente, G., Ballottari, M., Truong, T.B., Morosinotto, T., Ahn, T.K., Fleming, G.R., Niyogi, K.K., and Bassi, R. (2011). Analysis of LhcSR3, a protein essential for feedback de-excitation in the green alga *Chlamydomonas reinhardtii*. *PLoS Biol.* **9**:e1000577.

Bonente, G., Passarini, F., Cazzaniga, S., Mancone, C., Buia, M.C., Tripodi, M., Bassi, R., and Caffarri, S. (2008). The occurrence of the psbS gene product in *Chlamydomonas reinhardtii* and in other photosynthetic organisms and its correlation with energy quenching. *Photochem. Photobiol.* **84**:1359-1370.

Bonente, G., Pippa, S., Castellano, S., Bassi, R., and Ballottari, M. (2012). Acclimation of *Chlamydomonas reinhardtii* to different growth irradiances. *J. Biol. Chem.* **287**:5833-5847.

Boschetti, A. and Schmid, K. (1998). Energy supply for ATP-synthase deficient chloroplasts of *Chlamydomonas reinhardii*. *Plant Cell Physiol.* **39**:160-168.

Brautigam, K., Dietzel, L., Kleine, T., Stroher, E., Wormuth, D., Dietz, K.J., Radke, D., Wirtz, M., Hell, R., Dormann, P., et al. (2009). Dynamic plastid redox signals integrate gene expression and metabolism to induce distinct metabolic states in photosynthetic acclimation in *Arabidopsis*. *Plant Cell* **21**:2715-2732.

Brautigam, K., Dietzel, L., and Pfannschmidt, T. (2010). Hypothesis: A binary redox control mode as universal regulator of photosynthetic light acclimation. *Plant Signal Behav.* **5**:81-85.

Brune, I., Barzantny, H., Klötzel, M., Jones, J., James, G., and Tauch, A. (2011). Identification of McbR as transcription regulator of aecD and genes involved in methionine and cysteine biosynthesis in Corynebacterium jeikeium K411. *J. Biotech.* **151**:22-29.

Brzezowski, P., Schlicke, H., Richter, A., Dent, R.M., Niyogi, K.K., and Grimm, B. (2014). The GUN4 protein plays a regulatory role in tetrapyrrole biosynthesis and chloroplast-to-nucleus signalling in *Chlamydomonas reinhardtii*. *Plant J.* **79**:285-298.

Büchel, C. (2015). Evolution and function of light harvesting proteins. *J. Plant Physiol.* **172**:62-75.

Bulté, L., Gans, P., Rebéillé, F., and Wollman, F.A. (1990). ATP control on state transitions *in vivo* in *Chlamydomonas reinhardtii*. *BBA-Bioenergetics* **1020**:72-80.

Caffarri, S., Kouril, R., Kereiche, S., Boekema, E.J., and Croce, R. (2009). Functional architecture of higher plant photosystem II supercomplexes. *EMBO J.* **28**:3052-3063.

Cao, M., Fu, Y., Guo, Y., and Pan, J. (2009). *Chlamydomonas* (Chlorophyceae) colony PCR. *Protoplasma* **235**:107-110.

Cardol, P., Alric, J., Girard-Bascou, J., Franck, F., Wollman, F.A., and Finazzi, G. (2009). Impaired respiration discloses the physiological significance of state transitions in *Chlamydomonas*. *Proc. Natl. Acad. Sci. USA* **106**:15979 - 15984.

Chen, Y.B., Durnford, D.G., Koblizek, M., and Falkowski, P.G. (2004). Plastid regulation of Lhcb1 transcription in the chlorophyte alga *Dunaliella tertiolecta*. *Plant Physiol.* **136**:3737-3750.

Chiaiese, P., Palomba, F., Tatino, F., Lanzillo, C., Pinto, G., Pollio, A., and Filippone, E. (2011). Engineered tobacco and microalgae secreting the fungal laccase PDXA1b reduce phenol content in olive oil mill wastewater. *Enzyme Microb. Tech.* **49**:540-546.

Choi, Y.-E., Rhee, J.-K., Kim, H.-S., Ahn, J.-W., Hwang, H., and Yang, J.-W. (2015). Chemical genetics approach reveals importance of cAMP and MAP kinase signaling for lipid and carotenoid biosynthesis in microalgae. *J. Microbiol. Biotechn.*

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156-159.

Chu, L. and Robinson, D.K. (2001). Industrial choices for protein production by large-scale cell culture. *Curr. Opin. Biotech.* **12**:180-187.

Cieśla, J. (2006). Metabolic enzymes that bind RNA: yet another level of cellular regulatory network? *Acta Biochim. Pol.* **53**:11-32.

Cohen, I., Sapir, Y., and Shapira, M. (2006). A conserved mechanism controls translation of rubisco large subunit in different photosynthetic organisms. *Plant Phyiol.* **141**:1089-1097.

Cohen, S.N., Chang, A.C.Y., Boyer, H.W., and Helling, R.B. (1973). Construction of biologically functional bacterial plasmids *in-vitro*. *Proc. Natl. Acad. Sci. USA* **70**:3240-3244.

Corpas, F.J., Palma, J.M., Del Río, L.A., and Barroso, J.B. (2009). Evidence supporting the existence of l-arginine-dependent nitric oxide synthase activity in plants. *New Phytol.* **184**:9-14.

Cournac, L., Latouche, G., Cerovic, Z., Redding, K., Ravenel, J., and Peltier, G. (2002). In vivo interactions between photosynthesis, mitorespiration, and chlororespiration in *Chlamydomonas reinhardtii*. *Plant Physiol.* **129**:1921-1928.

Croce, R. and van Amerongen, H. (2014). Natural strategies for photosynthetic light harvesting. *Nat. Chem. Biol.* **10**:492-501.

Croce, R., Weiss, S., and Bassi, R. (1999). Carotenoid-binding Sites of the Major Light-harvesting Complex II of Higher Plants. *J. Biol. Chem.* **274**:29613-29623.

Dainese, P. and Bassi, R. (1991). Subunit stoichiometry of the chloroplast photosystem-II antenna system and aggregation state of the component chlorophyll-a/b binding-proteins. *J. Biol. Chem.* **266**:8136-8142.

Dall'Osto, L., Ünlü, C., Cazzaniga, S., and van Amerongen, H. (2014). Disturbed excitation energy transfer in *Arabidopsis thaliana* mutants lacking minor antenna complexes of photosystem II. *BBA-Bioenergetics* **1837**:1981-1988.

Daloso, D.M., Müller, K., Obata, T., Florian, A., Tohge, T., Bottcher, A., Riondet, C., Bariat, L., Carrari, F., Nunes-Nesi, A., et al. (2015). Thioredoxin, a master regulator of the tricarboxylic acid cycle in plant mitochondria. *Proc. Natl. Acad. Sci. USA* **112**:E1392-E1400.

Dang, K.-V., Plet, J., Tolleter, D., Jokel, M., Cuiné, S., Carrier, P., Auroy, P., Richaud, P., Johnson, X., Alric, J., et al. (2014). Combined increases in mitochondrial cooperation and oxygen photoreduction compensate for deficiency in cyclic electron flow in *Chlamydomonas reinhardtii*. *Plant Cell* **26**:3036-3050.

Danon, A. and Mayfield, S.P. (1994). Light-regulated translation of chloroplast messenger RNAs through redox potential. *Science* **266**:1717-1719.

Davies, J.P., Weeks, D.P., and Grossman, A.R. (1992). Expression of the arylsulfatase gene from the beta-2-tubulin promoter in *Chlamydomonas reinhardtii*. *Nucleic Acids Res.* **20**:2959-2965.

Davis, M.C., Fiehn, O., and Durnford, D.G. (2013). Metabolic acclimation to excess light intensity in *Chlamydomonas reinhardtii*. *Plant Cell Environ.* **36**:1391-1405.

Del Río, L.A., Corpas, F.J., Sandalio, L.M., Palma, J.M., and Barroso, J.B. (2003). Plant peroxisomes, reactive oxygen metabolism and nitric oxide. *IUBMB life* **55**:71-81.

Delosme, R., Olive, J., and Wollman, F.A. (1996). Changes in light energy distribution upon state transitions: An in vivo photoacoustic study of the wild type and photosynthesis mutants from *Chlamydomonas reinhardtii*. *BBA-Bioenergetics* **1273**:150-158.

Demerec, M. and Latarjet, R. (1946). Mutations in bacteria induced by radiations. *Cold S. H. Q. B.* **11**:38-50.

Depège, N., Bellafiore, S., and Rochaix, J.D. (2003). Role of chloroplast protein kinase STT7 in LHCII phosphorylation and state transition in *Chlamydomonas*. *Science* **299**:1572-1575.

Dietz, K.-J. (2015). Efficient high light acclimation involves rapid processes at multiple mechanistic levels. *J. Exp. Bot.* doi 10.1093/jxb/eru505.

Dietz, K.-J., Schreiber, U., and Heber, U. (1985). The relationship between the redox state of Q_A and photosynthesis in leaves at various carbon-dioxide, oxygen and light regimes. *Planta* **166**:219-226.

Ding, J., Li, X., and Hu, H. (2012). Systematic prediction of *cis*-regulatory elements in the *Chlamydomonas reinhardtii* genome using comparative genomics. *Plnat physiol.* **160**:613-623.

Doran, P.M. (2000). Foreign protein production in plant tissue cultures. *Curr. Opin. Biotech.* **11**:199-204.

Drop, B., Webber-Birungi, M., Fusetti, F., Kouril, R., Redding, K.E., Boekema, E.J., and Croce, R. (2011). Photosystem I of *Chlamydomonas reinhardtii* contains nine light-harvesting complexes (Lhca) located on one side of the core. *J. Biol. Chem.* **286**:44878-44887.

Drop, B., Webber-Birungi, M., Yadav, S.K.N., Filipowicz-Szymanska, A., Fusetti, F., Boekema, E.J., and Croce, R. (2014a). Light-harvesting complex II (LHCII) and its supramolecular organization in *Chlamydomonas reinhardtii*. *BBA-Bioenergetics* **1837**:63-72.

Drop, B., Yadav K. N, S., Boekema, E.J., and Croce, R. (2014b). Consequences of state transitions on the structural and functional organization of photosystem I in the green alga *Chlamydomonas reinhardtii*. *Plant J.* **78**:181-191.

Dunn, M.A., White, A.J., Vural, S., and Hughes, M.A. (1998). Identification of promoter elements in a low-temperature-responsive gene (blt4.9) from barley (*Hordeum vulgare* L.). *Plant. Mol. Biol.* **38**:551-564.

Dunsmuir, P., Smith, S., and Bedbrook, J. (1982). The major chlorophyll a/b binding protein of petunia is composed of several polypeptides encoded by a number of distinct nuclear genes. *J. Mol. Appl. Genet.* **2**:285-300.

Durnford, D.G., Price, J.A., McKim, S.M., and Sarchfield, M.L. (2003). Light-harvesting complex gene expression is controlled by both transcriptional and post-transcriptional mechanisms during photoacclimation in *Chlamydomonas reinhardtii*. *Physiol. Plant.* **118**:193-205.

Eichler-Stahlberg, A., Weisheit, W., Ruecker, O., and Heitzer, M. (2009). Strategies to facilitate transgene expression in *Chlamydomonas reinhardtii*. *Planta* **229**:873-883.

Elrad, D. and Grossman, A.R. (2004). A genome's-eye view of the light-harvesting polypeptides of *Chlamydomonas reinhardtii*. *Curr. Genet.* **45**:61-75.

Elrad, D., Niyogi, K.K., and Grossman, A.R. (2002). A major light-harvesting polypeptide of photosystem II functions in thermal dissipation. *Plant Cell* **14**:1801-1816.

Ensminger, I., Busch, F., and Huner, N. (2006). Photostasis and cold acclimation: sensing low temperature through photosynthesis. *Physiol. Plantarum* **126**:28-44.

Erickson, E., Wakao, S., and Niyogi, K.K. (2015). Light stress and photoprotection in *Chlamydomonas reinhardtii*. *Plant J.* doi 10.1111/tpj.12825.

Escoubas, J.M., Lomas, M., LaRoche, J., and Falkowski, P.G. (1995). Light intensity regulation of cab gene transcription is signaled by the redox state of the plastoquinone pool. *Proc. Natl. Acad. Sci. USA* **92**:10237-10241.

Fackelmayer, F.O. (2005). Protein arginine methyltransferases: guardians of the Arg? *Trends Biochem. Sci.* **30**:666-671.

Falk, S. and Palmqvist, K. (1992). Photosynthetic light utilization efficiency, photosystem II heterogeneity, and fluorescence quenching in *Chlamydomonas reinhardtii* during the induction of the CO_2 concentrating mechanism. *Plant Physiol.* **100**:685-691.

Falkowski, P.G. and Owens, T.G. (1980). Light-shade adaptation two strategies in marine phytoplankton. *Plant Physiol.* **66**:592-595.

Fang, W., Si, Y., Douglass, S., Casero, D., Merchant, S.S., Pellegrini, M., Ladunga, I., Liu, P., and Spalding, M.H. (2012). Transcriptome-wide changes in *Chlamydomonas reinhardtii* gene expression regulated by carbon dioxide and the CO_2-concentrating mechanism regulator CIA5/CCM1. *Plant Cell* **24**:1876-1893.

Feechan, A., Kwon, E., Yun, B.-W., Wang, Y., Pallas, J.A., and Loake, G.J. (2005). A central role for S-nitrosothiols in plant disease resistance. *Proc. Natl. Acad. Sci. USA* **102**:8054-8059.

Feinleib, M.E.H. and Curry, G.M. (1971). The relationship between stimulus intensity and oriented phototactic response (topotaxis) in *Chlamydomonas*. *Physiol. Plantarum* **25**:346-352.

Ferrante, P., Ballottari, M., Bonente, G., Giuliano, G., and Bassi, R. (2012). The LHCBM1 and LHCBM2/7 polypeptides, components of the major LHCII complex, have distinct functional roles in the photosynthetic antenna system of *Chlamydomonas reinhardtii*. *J. Biol. Chem.* **287**:16276-16288.

Fett, J.P. and Coleman, J.R. (1994). Regulation of periplasmic carbonic anhydrase expression in *Chlamydomonas reinhardtii* by acetate and pH. *Plant Physiol.* **106**:103-108.

Fey, V., Wagner, R., Bräutigam, K., and Pfannschmidt, T. (2005). Photosynthetic redox control of nuclear gene expression. *J. Exp. Bot.* **56**:1491-1498.

Finazzi, G., Johnson, G.N., Dall'Osto, L., Zito, F., Bonente, G., Bassi, R., and Wollman, F.-A. (2006). Nonphotochemical quenching of chlorophyll fluorescence in *Chlamydomonas reinhardtii*. *Biochemistry* **45**:1490-1498.

Finazzi, G., Rappaport, F., Furia, A., Fleischmann, M., Rochaix, J.D., Zito, F., and Forti, G. (2002). Involvement of state transitions in the switch between linear and cyclic electron flow in *Chlamydomonas reinhardtii*. *EMBO Rep.* **3**:280-285.

Fischer, B., Dayer, R., Schwarzenbach, Y., Lemaire, S., Behra, R., Liedtke, A., and Eggen, R. (2009). Function and regulation of the glutathione peroxidase homologous gene *GPXH/GPX5* in *Chlamydomonas reinhardtii*. *Plant Mol. Biol.* **71**:569-583.

Fischer, B.B., Hideg, E., and Liszkay, A. (2013). Production, detection and signaling of singlet oxygen in photosynthetic organisms. *Antioxid. Redox Sign.* **18**:2145-2162.

Fischer, B.B., Eggen, R.I.L., Trebst, A., and Krieger-Liszkay, A. (2006). The glutathione peroxidase homologous gene *Gpxh* in *Chlamydomonas reinhardtii* is upregulated by singlet oxygen produced in photosystem II. *Planta* **223**:583-590.

Fischer, B.B., Krieger-Liszkay, A., and Eggen, R.I.L. (2005). Oxidative stress induced by the photosensitizers neutral red (type I) or rose bengal (type II) in the light causes different molecular responses in *Chlamydomonas reinhardtii*. *Plant Sci.* **168**:747-759.

Fischer, R., Stoger, E., Schillberg, S., Christou, P., and Twyman, R.M. (2004). Plant-based production of biopharmaceuticals. *Curr. Opin. Plant Biol.* **7**:152-158.

Fleischmann, M.M., Ravanel, S., Delosme, R., Olive, J., Zito, F., Wollman, F.A., and Rochaix, J.D. (1999). Isolation and characterization of photoautotrophic mutants of *Chlamydomonas reinhardtii* deficient in state transition. *J. Biol. Chem.* **274**:30987-30994.

Floris, M., Bassi, R., Robaglia, C., Alboresi, A., and Lanet, E. (2013). Post-transcriptional control of light-harvesting genes expression under light stress. *Plant Mol. Biol.* **82**:147-154.

Foresi, N., Correa-Aragunde, N., Parisi, G., Caló, G., Salerno, G., and Lamattina, L. (2010). Characterization of a nitric oxide synthase from the plant kingdom: NO generation from the green alga *Ostreococcus tauri* is light irradiance and growth phase dependent. *Plant Cell* **22**:3816-3830.

Formighieri, C., Ceol, M., Bonente, G., Rochaix, J.-D., and Bassi, R. (2012). Retrograde signaling and photoprotection in a gun4 mutant of *Chlamydomonas reinhardtii*. *Mol. Plant* **5**:1242–1262.

Foyer, C.H. and Noctor, G. (2009). Redox regulation in photosynthetic organisms: signaling, acclimation, and practical implications. *Antioxid. Redox Sign.* **11**:861-905.

Frank, H.A., Cua, A., Chynwat, V., Young, A., Gosztola, D., and Wasielewski, M.R. (1994). Photophysics of the carotenoids associated with the xanthophyll cycle in photosynthesis. *Photosynth. Res.* **41**:389-395.

Franklin, S.E. and Mayfield, S.P. (2004). Prospects for molecular farming in the green alga *Chlamydomonas reinhardtii*. *Curr. Opin. Plant Biol.* **7**:159-165.

Frigerio, S., Campoli, C., Zorzan, S., Fantoni, L.I., Crosatti, C., Drepper, F., Haehnel, W., Cattivelli, L., Morosinotto, T., and Bassi, R. (2007). Photosynthetic antenna size in higher plants is controlled by the plastoquinone redox state at the post-transcriptional rather than transcriptional level. *J. Biol. Chem.* **282**:29457-29469.

Fröhlich, A. and Durner, J. (2011). The hunt for plant nitric oxide synthase (NOS): is one really needed? *Plant Sci.* **181**:401-404.

Fuhrmann, M., Hausherr, A., Ferbitz, L., Schödl, T., Heitzer, M., and Hegemann, P. (2004). Monitoring dynamic expression of nuclear genes in *Chlamydomonas reinhardtii* by using a synthetic luciferase reporter gene. *Plant Mol. Biol.* **55**:869-881.

Fujita, Y., Iwama, Y., Ohki, K., Murakami, A., and Hagiwara, N. (1989). Regulation of the size of light-harvesting antennae in response to light intensity in the green alga *Chlorella pyrenoidosa*. *Plant Cell Physiol.* **30**:1029-1037.

Fukuzawa, H., Fujiwara, S., Yamamoto, Y., Dionisio-Sese, M.L., and Miyachi, S. (1990). cDNA cloning, sequence, and expression of carbonic anhydrase in *Chlamydomonas reinhardtii*: regulation by environmental CO_2 concentration. *Proc. Natl. Acad. Sci. USA* **87**:4383-4387.

Fukuzawa, H., Miura, K., Ishizaki, K., Kucho, K.-i., Saito, T., Kohinata, T., and Ohyama, K. (2001). CCM1, a regulatory gene controlling the induction of a carbon-concentrating mechanism in *Chlamydomonas reinhardtii* by sensing CO_2 availability. *Proc. Natl. Acad. Sci. USA* **98**:5347-5352.

Gagné, G. and Guertin, M. (1992). The early genetic response to light in the green unicellular alga *Chlamydomonas eugametos* grown under light/dark cycles involves genes that represent direct responses to light and photosynthesis. *Plant Mol. Biol.* **18**:429-445.

Gahan, C.G. (2012). The bacterial lux reporter system: applications in bacterial localisation studies. *Curr. Gene Ther.* **12**:12-19.

Gans, P. and Rebeille, F. (1990). Control in the dark of the plastoquinone redox state by mitchondrial anctivity in *Chlamydomonas reinhardtii*. *BBA-Bioenergetics* **1015**:150-155.

Gasteiger, E., Hoogland, C., Gattiker, A., Wilkins, M.R., Appel, R.D., and Bairoch, A. (2005). Protein identification and analysis tools on the ExPASy server. In: The proteomics protocols handbook Springer. 571-607.

Geigenberger, P. and Fernie, A.R. (2014). Metabolic control of redox and redox control of metabolism in plants. *Antioxid. Redox Sign.* **17**:1866-1875

Gomord, V., Fitchette, A.-C., Menu-Bouaouiche, L., Saint-Jore-Dupas, C., Plasson, C., Michaud, D., and Faye, L. (2010). Plant-specific glycosylation patterns in the context of therapeutic protein production. *Plant Biotech. J.* **8**:564-587.

Gordillo, F.J., Jiménez, C., Chavarría, J., and Niell, F.X. (2001). Photosynthetic acclimation to photon irradiance and its relation to chlorophyll fluorescence and carbon assimilation in the halotolerant green alga *Dunaliella viridis*. *Photosynth. Res.* **68**:225-235.

Gorman, D.S. and Levine, R.P. (1965). Cytochrome f and plastocyanin - their sequence in photosynthetic electron transport chain of *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. USA* **54**:1665-1669.

Gould, N., Doulias, P.-T., Tenopoulou, M., Raju, K., and Ischiropoulos, H. (2013). Regulation of protein function and signaling by reversible cysteine S-nitrosylation. *J. Biol. Chem.* **288**:26473-26479.

Goyer, A., Decottignies, P., Lemaire, S., Ruelland, E., Issakidis-Bourguet, E., Jacquot, J.-P., and Miginiac-Maslow, M. (1999). The internal Cys-207 of sorghum leaf NADP-malate dehydrogenase can form mixed disulphides with thioredoxin. *FEBS Lett.* **444**:165-169.

Graumann, P.L. and Marahiel, M.A. (1998). A superfamily of proteins that contain the cold-shock domain. *Trends Biochem. Sci.* **23**:286-290.

Grek, C.L., Zhang, J., Manevich, Y., Townsend, D.M., and Tew, K.D. (2013). Causes and consequences of cysteine S-glutathionylation. *J. Biol. Chem.* **288**:26497-26504.

Grewe, S., Ballottari, M., Alcocer, M., D'Andrea, C., Blifernez-Klassen, O., Hankamer, B., Mussgnug, J.H., Bassi, R., and Kruse, O. (2014). Light-harvesting complex protein LHCBM9 is critical for photosystem II activity and hydrogen production in *Chlamydomonas reinhardtii*. *Plant Cell* **26**:1598-1611.

Grimm, B., Dehesh, K., Zhang, L., and Leister, D. (2014). Intracellular Communication. *Mol. Plant* **7**:1071-1074.

Grossman, A.R., Lohr, M., and Im, C.S. (2004). *Chlamydomonas reinhardtii* in the landscape of pigments. *Annu. Rev. Genet.* **38**:119-173.

Guex, N. and Peitsch, M.C. (1997). Swiss-model and the Swiss-Pdb Viewer: an environment for comparative protein modeling. *Electrophoresis* **18**:2714-2723.

Gutu, A., Nesbit, A.D., Alverson, A.J., Palmer, J.D., and Kehoe, D.M. (2013). Unique role for translation initiation factor 3 in the light color regulation of photosynthetic gene expression. *Proc. Natl. Acad. Sci. USA.* **110**:16253-16258.

Hahn, D. and Kück, U. (1999). Identification of DNA sequences controlling light- and chloroplast-dependent expression of the *lhcb1* gene from *Chlamydomonas reinhardtii*. *Curr. Genet.* **34**:459-466.

Hallmann, A. (2007). Algal transgenics and biotechnology. *Transgenic Plant J* **1**:81-98.

Hara, M.R., Agrawal, N., Kim, S.F., Cascio, M.B., Fujimuro, M., Ozeki, Y., Takahashi, M., Cheah, J.H., Tankou, S.K., Hester, L.D., et al. (2005). S-nitrosylated GAPDH initiates apoptotic cell death by nuclear translocation following Siah1 binding. *Nat. Cell. Biol.* **7**:665-674.

Harris, E.H. (2009a). *Chlamydomonas* in the Laboratory. In: The Chlamydomonas Sourcebook: Introduction to *Chlamydomonas* and its Laboratory Use. Vol. 1, 2nd ed., Academic Press, San Diego. 242-245.

Harris, E.H. (2009b). The life of an acetate flagellate. In: The Chlamydomonas Sourcebook: Introduction to *Chlamydomonas* and its Laboratory Use. Vol.1, 2nd ed., Academic Press, San Diego. 159-210.

Havaux, M. and Niyogi, K.K. (1999). The violaxanthin cycle protects plants from photooxidative damage by more than one mechanism. *Proc. Natl. Acad. Sci. USA* **96**:8762-8767.

Hayashi, Y., Sato, N., Shinozaki, A., and Watanabe, M. (2014). Increase in peroxisome number and the gene expression of putative glyoxysomal enzymes in *Chlamydomonas* cells supplemented with acetate. *J. Plant Res.* **128**:177-185.

Hayashi, Y. and Shinozaki, A. (2012). Visualization of microbodies in *Chlamydomonas reinhardtii*. *J. Plant Res.* **125**:579-586.

Heifetz, P.B., Forster, B., Osmond, C.B., Giles, L.J., and Boynton, J.E. (2000). Effects of acetate on facultative autotrophy in *Chlamydomonas reinhardtii* assessed by photosynthetic measurements and stable isotope analyses. *Plant Physiol.* **122**:1439-1445.

Heitzer, M. and Zschoernig, B. (2007). Construction of modular tandem expression vectors for the green alga *Chlamydomonas reinhardtii* using the Cre/Iox-system. *Biotechniques* **43**:324.

Herrin, D.L., and Nickelsen, J. (2004). Chloroplast RNA processing and stability. *Photosynth. Res.* **82**:301-314.

Higo, K., Ugawa, Y., Iwamoto, M., and Korenaga, T. (1999). Plant cis-acting regulatory DNA elements (PLACE) database: 1999. *Nucl. Acids Res.* **27**:297-300.

Hoefnagel, M.H., Atkin, O.K., and Wiskich, J.T. (1998). *BBA-Bioenergetics* **1366**:235-255.

Horton, P. and Black, M.T. (1980). Activation of adenosine-5'- triphosphate induced quenching of chlorophyll fluorescence by reduced plastoquinone - The basis of state-I-stateII transitions in chloroplasts. *FEBS Lett.* **119**:141-144.

Humby, P.L., Cunningham, M.L., Saunders, H.L., Price, J.A., and Durnford, D.G. (2009). Compartmental cross-talk in the regulation of light harvesting complex transcription under short-term light and temperature stress in *Chlamydomonas reinhardtii*. *Botany* **87**:375-386.

Huner, N., Öquist, G., and Sarhan, F. (1998). Energy balance and acclimation to light and cold. *Trends Plant Sci.* **3**:224-230.

Huppe, H.C., Picaud, A., Buchanan, B.B., and Miginiac-Maslow, M. (1991). Identification of an NADP/thioredoxin system in *Chlamydomonas reinhardtii*. *Planta* **186**:115-121.

Hutner, S., Provasoli, L., Schatz, A., and Haskins, C. (1950). Some approaches to the study of the role of metals in the metabolism of microorganisms. *P. Am. Philos. Soc.* **94**:152-170.

Hyams, J. and Davies, D.R. (1972). The induction and characterisation of cell wall mutants of *Chlamydomonas reinhardi*. *Mut. Res.* **14**:381-389.

Ikonomou, L., Schneider, Y.J., and Agathos, S.N. (2003). Insect cell culture for industrial production of recombinant proteins. *Appl. Microbiol. Biot.* **62**:1-20.

Im, C.-S., Eberhard, S., Huang, K., Beck, C.F., and Grossman, A.R. (2006). Phototropin involvement in the expression of genes encoding chlorophyll and carotenoid biosynthesis enzymes and LHC apoproteins in *Chlamydomonas reinhardtii*. *Plant J.* **48**:1-16.

Imbault, P., Wittemer, C., Johanningmeier, U., Jacobs, J.D., and Howell, S.H. (1988). Structure of the *Chlamydomonas reinhardtii cabII-1* gene encoding a chlorophyll-*a*/*b*-binding protein. *Gene* **73**:397-407.

Itakura, K., Hirose, T., Crea, R., Riggs, A.D., Heyneker, H.L., Bolivar, F., and Boyer, H.W. (1977). Expression in *Escherichia coli* of a chemically synthesized gene for the hormone somatostatin. *Science* **198**:1056-1063.

Iwai, M., Kato, N.,and Minagawa, J. (2007). Distinct physiological responses to a high light and low CO_2 environment revealed by fluorescence quenching in photoautotrophically grown *Chlamydomonas reinhardtii*. *Photosynth. Res.* **94**:307-314.

Iwai, M., Takahashi, Y., and Minagawa, J. (2008). Molecular remodeling of photosystem II during state transitions in *Chlamydomonas reinhardtii*. *Plant Cell* **20**:2177-2189.

Iwai, M., Takizawa, K., Tokutsu, R., Okamuro, A., Takahashi, Y., and Minagawa, J. (2010a). Isolation of the elusive supercomplex that drives cyclic electron flow in photosynthesis. *Nature* **464**:1210-1213.

Iwai, M., Yokono, M., Inada, N., and Minagawa, J. (2010b). Live-cell imaging of photosystem II antenna dissociation during state transitions. *Proc. Natl. Acad. Sci. USA* **107**:2337-2342.

Jacobshagen, S., Kindle, K.L., and Johnson, C.H. (1996). Transcription of CABII is regulated by the biological clock in *Chlamydomonas reinhardtii*. *Plant Mol. Biol.* **31**:1173-1184.

Jaffrey, S.R. and Snyder, S.H. (2001). The biotin switch method for the detection of S-nitrosylated proteins. *Sci. STKE* **2001**:pl1.

Jiang, C., Iu, B., and Singh, J. (1996). Requirement of a CCGAC *cis*-acting element for cold induction of the BN115 gene from winter *Brassica napus*. *Plant. Mol. Biol.* **30**:679-684.

Johanningmeier, U. (1988). Possible control of transcript levels by chlorophyll precursors in *Chlamydomonas*. *Eur. J. Biochem.* **177**:417-424.

Johanningmeier, U. and Howell, S.H. (1984). Regulation of light-harvesting chlorophyll-binding protein mRNA accumulation in *Chlamydomonas reinhardtii*. Possible involvement of chlorophyll synthesis precursors. *J. Biol. Chem.* **259**:13541-13549.

Johnson, M.P., Brain, A.P., and Ruban, A.V. (2011). Changes in thylakoid membrane thickness associated with the reorganization of photosystem II light harvesting complexes during photoprotective energy dissipation. *Plant Signal. Behav.* **6**:1386-1390.

Johnson, X. and Alric, J. (2012). Interaction between starch breakdown, acetate assimilation, and photosynthetic cyclic electron flow in *Chlamydomonas reinhardtii*. *J. Biol. Chem.* **287**:26445-26452.

Johnson, X. and Alric, J. (2013). Central carbon metabolism and electron transport in *Chlamydomonas reinhardtii*, metabolic constraints for carbon partitioning between oil and starch. *Eukaryot. Cell* **12**:776-793

Johnson, X., Steinbeck, J., Dent, R.M., Takahashi, H., Richaud, P., Ozawa, S.-I., Houille-Vernes, L., Petroutsos, D., Rappaport, F., Grossman, A.R., et al. (2014). Proton gradient regulation 5-mediated cyclic electron flow under ATP- or redox-limited conditions: A study of ΔATPase pgr5 and ΔrbcL pgr5 mutants in the green alga *Chlamydomonas reinhardtii*. *Plant Physiol.* **165**:438-452.

Kadonaga, J.T. (2012). Perspectives on the RNA polymerase II core promoter. *Dev. Biol.* **1**:40-51.

Kargul, J., Nield, J., and Barber, J. (2003). Three-dimensional reconstruction of a light-harvesting complex I-photosystem I (LHCI-PSI) supercomplex from the green alga *Chlamydomonas reinhardtii* - Insights into light-harvesting for PSI. *J. Biol. Chem.* **278**:16135-16141.

Kim, H.J., Kim, Y.K., Park, J.Y., and Kim, J. (2002). Light signalling mediated by phytochrome plays an important role in cold-induced gene expression through the C-repeat/dehydration responsive element (C/DRE) in *Arabidopsis thaliana*. *Plant J.* **29**:693-704.

Kindle, K.L. (1987). Expression of a gene for a light-harvesting chlorophyll a/b-binding protein in *Chlamydomonas reinhardtii*: effect of light and acetate. *Plant Mol. Biol.* **9**:547-563.

Kindle, K.L. (1990). High-frequency nuclear transformation of *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. USA* **87**:1228-1232.

Kirst, H., Formighieri, C., and Melis, A. (2014). Maximizing photosynthetic efficiency and culture productivity in cyanobacteria upon minimizing the phycobilisome light-harvesting antenna size. *BBA-Bioenergetics* **1837**:1653-1664.

Kirst, H., Garcia-Cerdan, J.G., Zurbriggen, A., Ruehle, T., and Melis, A. (2012). Truncated photosystem chlorophyll antenna size in the green microalga *Chlamydomonas reinhardtii* upon deletion of the *TLA3-CpSRP43* gene. *Plant Physiol.* **160**:2251-2260.

Kleine, T., Voigt, C., and Leister, D. (2009). Plastid signalling to the nucleus: messengers still lost in the mists? *Trends Genet.* **25**:185-192.

Klepper, L. (1979). Nitric oxide (NO) and nitrogen dioxide (NO_2) emissions from herbicide-treated soybean plants. *Atmos. Environ.* **13**:537-542.

Kneeshaw, S., Gelineau, S., Tada, Y., Loake, Gary J., and Spoel, Steven H. (2014). Selective protein denitrosylation activity of thioredoxin-h5 modulates plant immunity. *Mol. Cell* **56**:153-162.

Kong, Q.-X., Li, L., Martinez, B., Chen, P., and Ruan, R. (2010). Culture of microalgae *Chlamydomonas reinhardtii* in wastewater for biomass feedstock production. *Appl. Biochem. Biotech.* **160**:9-18.

König, J., Muthuramalingam, M., and Dietz, K.-J. (2012). Mechanisms and dynamics in the thiol/disulfide redox regulatory network: transmitters, sensors and targets. *Curr. Opin. Plant Biol.* **15**:261-268.

Koprivova, A., Altmann, F., Gorr, G., Kopriva, S., Reski, R., and Decker, E.L. (2003). N-glycosylation in the moss *Physcomitrella patens* is organized similarly to that in higher plants. *Plant Biol.* **5**:582-591.

Kouril, R., Dekker, J.P., and Boekema, E.J. (2012). Supramolecular organization of photosystem II in green plants. *BBA-Bioenergetics* **1817**:2-12.

Koussevitzky, S., Nott, A., Mockler, T.C., Hong, F., Sachetto-Martins, G., Surpin, M., Lim, J., Mittler, R., and Chory, J. (2007). Signals from chloroplasts converge to regulate nuclear gene expression. *Science* **316**:715-719.

Kovács, L., Wiessner, W., Kis, M., Nagy, F., Mende, D., and Demeter, S. (2000). Short- and long-term redox regulation of photosynthetic light energy distribution and photosystem stoichiometry by acetate metabolism in the green alga *Chlamydobotrys stellata*. *Photosynth. Res.* **65**:231-247.

Kropat, J., Tottey, S., Birkenbihl, R.P., Depege, N., Huijser, P., and Merchant, S. (2005). A regulator of nutritional copper signaling in *Chlamydomonas* is an SBP domain protein that recognizes the GTAC core of copper response element. *Proc. Natl. Acad. Sci. USA* **102**:18730-18735.

Kruse, O., Nixon, P.J., Schmid, G.H., and Mullineaux, C.W. (1999). Isolation of state transition mutants of *Chlamydomonas reinhardtii* by fluorescence video imaging. *Photosynth. Res.* **61**:43-51.

Kucho, K.-i., Ohyama, K., and Fukuzawa, H. (1999). CO_2-responsive transcriptional regulation of *CAH1* encoding carbonic anhydrase is mediated by enhancer and silencer regions in *Chlamydomonas reinhardtii*. *Plant Physiol.* **121**:1329-1337.

Kucho, K.-I., Yoshioka, S., Taniguchi, F., Ohyama, K., and Fukuzawa, H. (2003). *Cis*-acting elements and DNA-binding proteins involved in CO_2-responsive transcriptional activation of *CAH1* encoding a periplasmic carbonic anhydrase in *Chlamydomonas reinhardtii*. *Plant Physiol.* **133**:783-793.

Kühlbrandt, W., Wang, D.N., and Fujiyoshi, Y. (1994). Atomic model of plant light-harvesting complex by electron crystallography. *Nature* **367**:614-621.

Kukuruzinska, M.A. and Lennon, K. (1998). Protein N-glycosylation: Molecular genetics and functional significance. *Crit. Rev. Oral Biol. M.* **9**:415-448.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680-685.

Lamotte, O., Bertoldo, J.B., Besson-Bard, A., Rosnoblet, C., Aimé, S., Hichami, S., Terenzi, H., and Wendehenne, D. (2015). Protein S-nitrosylation: specificity and identification strategies in plants. *Front. Chem.* **2**:114.

Lauersen, K.J., Berger, H., Mussgnug, J.H., and Kruse, O. (2013). Efficient recombinant protein production and secretion from nuclear transgenes in *Chlamydomonas reinhardtii*. *J. Biotech.* **167**:101-110.

Le Quiniou, C., Tian, L., Drop, B., Wientjes, E., van Stokkum, I., van Oort, B., and Croce, R. (2015). PSI–LHCI of *Chlamydomonas reinhardtii*: Increasing the absorption cross section without losing efficiency. *BBA-Bioenergetics* **1847**:458-467.

Leisinger, U., Rüfenacht, K., Fischer, B., Pesaro, M., Spengler, A., Zehnder, A.J.B., and Eggen, R.I.L. (2001). The glutathione peroxidase homologous gene from *Chlamydomonas reinhardtii* is transcriptionally up-regulated by singlet oxygen. *Plant Mol. Biol.* **46**:395-408.

Leister, D. (2012). Retrograde signaling in plants: from simple to complex scenarios. *Front. Plant Sci.* **3**:135.

Lemaire, S. and Miginiac-Maslow, M. (2004). The thioredoxin superfamily in *Chlamydomonas reinhardtii*. *Photosynth. Res.* **82**:203-220.

Lemeille, S. and Rochaix, J.D. (2010). State transitions at the crossroad of thylakoid signalling pathways. *Photosynth. Res.* **106**:33-46.

Lemeille, S., Turkina, M.V., Vener, A.V., and Rochaix, J.-D. (2010). STT7-dependent phosphorylation during state transitions in the green alga *Chlamydomonas reinhardtii*. *Mol. Cell Proteomics* **9**:1281-1295.

Lemeille, S., Willig, A., Depège-Fargeix, N., Delessert, C., Bassi, R., and Rochaix, J.-D. (2009). Analysis of the chloroplast protein kinase STT7 during state transitions. *PLoS Biol.* **7**:664-675.

Lepistö, A., Toivola, J., Nikkanen, L., and Rintamäki, E. (2012). Retrograde signaling from functionally heterogeneous plastids. *Front. Plant Sci.* **3**:Nr. 286.

Lescot, M., Déhais, P., Thijs, G., Marchal, K., Moreau, Y., Van de Peer, Y., Rouzé, P., and Rombauts, S. (2002). PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucl. Acids Res.* **30**:325-327.

Lewin, R.A. (1952). Ultraviolet induced mutations in *Chlamydomonas moewusii* Gerloff. *J. Gen. Microbiol.* **6**:233-&.

Lindermayr, C., Saalbach, G., Bahnweg, G., and Durner, J. (2006). Differential inhibition of *Arabidopsis* methionine adenosyltransferases by protein S-nitrosylation. *J. Biol. Chem.* **281**:4285-4291.

Liu, J.-X. and Howell, S.H. (2010). Endoplasmic reticulum protein quality control and its relationship to environmental stress responses in plants. *Plant Cell* **22**:2930-2942.

Liu, L., Hausladen, A., Zeng, M., Que, L., Heitman, J., and Stamler, J.S. (2001). A metabolic enzyme for S-nitrosothiol conserved from bacteria to humans. *Nature* **410**:490-494.

Liu, Z.F., Yan, H.C., Wang, K.B., Kuang, T.Y., Zhang, J.P., Gui, L.L., An, X.M., and Chang, W.R. (2004). Crystal structure of spinach major light-harvesting complex at 2.72 angstrom resolution. *Nature* **428**:287-292.

Lodha, M., Schulz-Raffelt, M., and Schroda, M. (2008). A new assay for promoter analysis in *Chlamydomonas* reveals roles for heat shock elements and the TATA box in *HSP70A* promoter-mediated activation of transgene expression. *Eukaryot. Cell* **7**:172-176.

Loppes, R. and Radoux, M. (2001). Identification of short promoter regions involved in the transcriptional expression of the nitrate reductase gene in *Chlamydomonas reinhardtii*. *Plant Mol. Biol.* **45**:215-227.

Lucker, B.F. and Kramer, D.M. (2013). Regulation of cyclic electron flow in *Chlamydomonas reinhardtii* under fluctuating carbon availability. *Photosynth. Res.* **117**:449-459.

Lumbreras, V., Stevens, D.R., and Purton, S. (1998). Efficient foreign gene expression in *Chlamydomonas reinhardtii* mediated by an endogenous intron. *Plant J.* **14**:441-447.

Majmudar, J.D. and Martin, B.R. (2014). Strategies for profiling native S-nitrosylation. *Biopolymers* **101**:173-179.

Mamedov, T. and Yusibov, V. (2011). Green algae *Chlamydomonas reinhardtii* possess endogenous sialylated N-glycans. *FEBS Open Bio* **1**:15-22.

Manuell, A.L., Beligni, M.V., Elder, J.H., Siefker, D.T., Tran, M., Weber, A., McDonald, T.L., and Mayfield, S.P. (2007). Robust expression of a bioactive mammalian protein in *Chlamydomonas* chloroplast. *Plant Biotech. J.* **5**:402-412.

Martínez-Ruiz, A., Araújo, I.M., Izquierdo-Álvarez, A., Hernansanz-Agustín, P., Lamas, S., and Serrador, J.M. (2012). Specificity in S-nitrosylation: A short-range mechanism for NO signaling? *Antioxid. Redox Sign.* **19**:1220-1235.

Maxwell, D.P., Laudenbach, D.E., and Huner, N.P. (1995). Redox regulation of light-harvesting complex II and cab mRNA abundance in *Dunaliella salina*. *Plant Physiol.* **109**:787-795.

Maxwell, K. and Johnson, G.N. (2000). Chlorophyll fluorescence - a practical guide. *J. Exp. Bot.* **51**:659-668.

Mayfield, S.P., Manuell, A.L., Chen, S., Wu, J., Tran, M., Siefker, D., Muto, M., and Marin-Navarro, J. (2007). *Chlamydomonas reinhardtii* chloroplasts as protein factories. *Curr. Opin. Biotech.* **18**:126-133.

McKim, S.M. and Durnford, D.G. (2006). Translational regulation of light-harvesting complex expression during photo acclimation to high-light in *Chlamydomonas reinhardtii*. *Plant Physiol. Biochem.* **44**:857-865.

Mehler, A.H. (1951). Studies on reactions of illuminated chloroplasts: I. Mechanism of the reduction of oxygen and other hill reagents. *Arch. Biochem. Biophys.* **33**:65-77.

Melis, A. (2009). Solar energy conversion efficiencies in photosynthesis: minimizing the chlorophyll antennae to maximize efficiency. *Plant Sci.* **177**:272-280.

Melis, A., Neidhardt, J., and Benemann, J.R. (1998). *Dunaliella salina* (Chlorophyta) with small chlorophyll antenna sizes exhibit higher photosynthetic productivities and photon use efficiencies than normally pigmented cells. *J. Appl. Phycol.* **10**:515-525.

Merchant, S.S., Prochnik, S.E., Vallon, O., Harris, E.H., Karpowicz, S.J., Witman, G.B., Terry, A., Salamov, A., Fritz-Laylin, L.K., Maréchal-Drouard, L., et al. (2007). The *Chlamydomonas* genome reveals the evolution of key animal and plant functions. *Science* **318**:245-250.

Michelet, L., Zaffagnini, M., Marchand, C., Collin, V., Decottignies, P., Tsan, P., Lancelin, J.-M., Trost, P., Miginiac-Maslow, M., and Noctor, G. (2005). Glutathionylation of chloroplast thioredoxin f is a redox signaling mechanism in plants. *Proc. Natl. Acad. Sci. USA* **102**:16478-16483.

Michelet, L., Zaffagnini, M., Vanacker, H., Le Maréchal, P., Marchand, C., Schroda, M., Lemaire, S.D., and Decottignies, P. (2008). *In vivo* targets of S-thiolation in *Chlamydomonas reinhardtii*. *J. Biol. Chem.* **283**:21571-21578.

Middleton, A.J., Marshall, C.B., Faucher, F., Bar-Dolev, M., Braslavsky, I., Campbell, R.L., Walker, V.K., and Davies, P.L. (2012). Antifreeze protein from freeze-tolerant grass has a beta-roll fold with an irregularly structured ice-binding site. *J. Mol. Biol.* **416**:713-724.

Minagawa, J. (2009). Light-harvesting proteins. In: The Chlamydomonas Sourcebook: Organellar and metabolic processes. Vol. 2, 2nd ed., --Stern, DB, ed. Academic Press, San Diego. 503-539.

Minagawa, J. (2011). State transitions - The molecular remodeling of photosynthetic supercomplexes that controls energy flow in the chloroplast. *BBA-Bioenergetics* **1807**:897-905.

Miret, J.A. and Munné-Bosch, S. (2015). Redox signaling and stress tolerance in plants: a focus on vitamin E. *Ann. NY Acad. Sci.* doi 10.1111/nyas.12639

Mitra, M., Kirst, H., Dewez, D., and Melis, A. (2012). Modulation of the light-harvesting chlorophyll antenna size in *Chlamydomonas reinhardtii* by TLA1 gene over-expression and RNA interference. *Phil. Trans. R. Soc. Lond. B* **367**:3430-3443.

Mohanta, T.K., Arora, P.K., Mohanta, N., Parida, P., and Bae, H. (2015). Identification of new members of the MAPK gene family in plants shows diverse conserved domains and novel activation loop variants. *BMC Genomics* **16**:58.

Moreau, M., Lindermayr, C., Durner, J., and Klessig, D.F. (2010). NO synthesis and signaling in plants – where do we stand? *Physiol. Plantarum* **138**:372-383.

Morisse, S., Zaffagnini, M., Gao, X.-H., Lemaire, S.D., and Marchand, C.H. (2014). Insight into protein S-nitrosylation in *Chlamydomonas reinhardtii*. *Antioxid. Redox Sign.* **21**:1271-1284.

Moroney, J., Jungnick, N., DiMario, R., and Longstreth, D. (2013). Photorespiration and carbon concentrating mechanisms: two adaptations to high O_2, low CO_2 conditions. *Photosynth. Res.* **117**:121-131.

Morosinotto, T., Bassi, R., Frigerio, S., Finazzi, G., Morris, E., and Barber, J. (2006). Biochemical and structural analyses of a higher plant photosystem II supercomplex of a photosystem I-less mutant of barley. *FEBS J.* **273**:4616-4630.

Müller, F.W., Igloi, G.L., and Beck, C.F. (1992). Structure of a gene encoding heat-shock protein HSP70 from the unicellular alga *Chlamydomonas reinhardtii*. *Gene* **111**:165-173.

Murata, N. (1969). Control of excitation transfer in photosynthesis. I. Light-induced change of chlorophyll a fluoresence in *Porphyridium cruentum*. *BBA-Bioenergetics* **172**:242-251.

Murik, O., Elboher, A., and Kaplan, A. (2014). Dehydroascorbate: a possible surveillance molecule of oxidative stress and programmed cell death in the green alga *Chlamydomonas reinhardtii*. *New Phytol.* **202**:471-484.

Mussgnug, J.H., Thomas Hall, S., Rupprecht, J., Foo, A., Klassen, V., McDowall, A., Schenk, P.M., Kruse, O., and Hankamer, B. (2007). Engineering photosynthetic light capture: impacts on improved solar energy to biomass conversion. *Plant Biotechnol. J.* **5**:802-814.

Mussgnug, J.H., Wobbe, L., Elles, I., Claus, C., Hamilton, M., Fink, A., Kahmann, U., Kapazoglou, A., Mullineaux, C.W., Hippler, M., et al. (2005). NAB1 is an RNA binding protein involved in the light-regulated differential expression of the light-harvesting antenna of *Chlamydomonas reinhardtii*. *Plant Cell* **17**:3409-3421.

Nagy, G., Ünnep, R., Zsiros, O., Tokutsu, R., Takizawa, K., Porcar, L., Moyet, L., Petroutsos, D., Garab, G., Finazzi, G., et al. (2014). Chloroplast remodeling during state transitions in *Chlamydomonas reinhardtii* as revealed by noninvasive techniques *in vivo*. *Proc. Nat. Acad. Sci. USA* **111**:5042-5047.

Natali, A. and Croce, R. (2015). Characterization of the major light-harvesting complexes (LHCBM) of the green alga *Chlamydomonas reinhardtii*. *PLoS ONE* **10** doi: 10.1371/journal.pone.0119211.

Neale, P.J. and Melis, A. (1986). Algal photosynthetic membrane complexes and the photosynthesis-irradiance curve: A comparison of light-adaptation responses in *Chlamydomonas reinhardtii* (Chlorophyta). *J. Phycol.* **22**:531-538.

Nematollahi, G., Kianianmomeni, A., and Hallmann, A. (2006). Quantitative analysis of cell-type specific gene expression in the green alga *Volvox carteri*. *BMC Genomics* **7**:Nr. 321.

Neupert, J., Karcher, D., and Bock, R. (2009). Generation of *Chlamydomonas strains* that efficiently express nuclear transgenes. *Plant J.* **57**:1140-1150.

Nickelsen, J. and Rengstl, B. (2013). Photosystem II assembly: from cyanobacteria to plants. *Annu. Rev. Plant Biol.* **64**:609-635.

Niyogi, K.K. (2009). Photoprotection and high light responses. In: The Chlamydomonas Sourcebook: Organellar and metabolic processes. Vol. 2, 2nd ed., --Stern, DB, ed. Academic Press, San Diego. 847-870.

Niyogi, K.K., Bjorkman, O., and Grossman, A.R. (1997). The roles of specific xanthophylls in photoprotection. *Proc. Natl. Acad. Sci. USA* **94**:14162-14167.

Niyogi, K.K. and Truong, T.B. (2013). Evolution of flexible non-photochemical quenching mechanisms that regulate light harvesting in oxygenic photosynthesis. *Curr. Opin. Plant. Biol.* **16**:307-314.

Oey, M., Ross, I.L., Stephens, E., Steinbeck, J., Wolf, J., Radzun, K.A., Kügler, J., Ringsmuth, A.K., Kruse, O., and Hankamer, B. (2013). RNAi knock-down of LHCBM1, 2 and 3 increases photosynthetic H_2 production efficiency of the green alga *Chlamydomonas reinhardtii*. *PLoS ONE* **8**:e61375.

Palmqvist, K., Sundblad, L.G., Wingsle, G., and Samuelsson, G. (1990). Acclimation of photosynthetic light reactions during induction of inorganic carbon accumulation in the green alga *Chlamydomonas reinhardtii*. *Plant Physiol.* **94**:357-366.

Park, H. and Hoober, J.K. (1997). Chlorophyll synthesis modulates retention of apoproteins of light-harvesting complex II by the chloroplast in *Chlamydomonas reinhardtii*. *Physiol. Plant.* **101**:135-142.

Paulsen, H. and Bogorad, L. (1988). Diurnal and circadian rhythms in the accumulation and synthesis of mRNA for the light-harvesting chlorophyll a/b-binding protein in tobacco. *Plant Physiol.* **88**:1104.

Paulsen, H., Rümler, U., and Rüdiger, W. (1990). Reconstitution of pigment-containing complexes from light-harvesting chlorophyll *a/b*-binding protein overexpressed in *Escherichia coli*. *Planta* **181**:204-211.

Peers, G., Truong, T.B., Ostendorf, E., Busch, A., Elrad, D., Grossman, A.R., Hippler, M., and Niyogi, K.K. (2009). An ancient light-harvesting protein is critical for the regulation of algal photosynthesis. *Nature* **462**:518-521.

Pérez-Pérez, M.E., Zaffagnini, M., Marchand, C.H., Crespo, J.L., and Lemaire, S.D. (2014). The yeast autophagy protease Atg4 is regulated by thioredoxin. *Autophagy* **10**:1953-1964.

Perrine, Z., Negi, S., and Sayre, R.T. (2012). Optimization of photosynthetic light energy utilization by microalgae. *Algal Res.* **1**:134-142.

Pesaresi, P., Hertle, A., Pribil, M., Kleine, T., Wagner, R., Strissel, H., Ihnatowicz, A., Bonardi, V., Scharfenberg, M., and Schneider, A. (2009). *Arabidopsis* STN7 kinase provides a link between short-and long-term photosynthetic acclimation. *Plant Cell* **21**:2402-2423.

Peter, G.F. and Thornber, J.P. (1991). Biochemical composition and organization of higher plant photosystem II light-harvesting pigment-proteins. *J. Bio. Chem.* **266**:16745-16754.

Petersen, T.N., Brunak, S., von Heijne, G., and Nielsen, H. (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat. Methods* **8**:785-786.

Petracek, M.E., Dickey, L.F., Huber, S.C., and Thompson, W.F. (1997). Light-regulated changes in abundance and polyribosome association of ferredoxin mRNA are dependent on photosynthesis. *Plant Cell* **9**:2291-2300.

Petracek, M.E., Dickey, L.F., Nguyen, T.T., Gatz, C., Sowinski, D.A., Allen, G.C., and Thompson, W.F. (1998). Ferredoxin-1 mRNA is destabilized by changes in photosynthetic electron transport. *Proc. Natl. Acad. Sci. USA* **95**:9009-9013.

Pfannschmidt, T. (2003). Chloroplast redox signals: how photosynthesis controls its own genes. *Trends Plant Sci.* **8**:33-41.

Pfannschmidt, T. (2010). Plastidial retrograde signalling - a true "plastid factor" or just metabolite signatures? *Trends Plant Sci.* **15**:427-435.

Pietrzykowska, M., Suorsa, M., Semchonok, D.A., Tikkanen, M., Boekema, E.J., Aro, E.-M., and Jansson, S. (2014). The Light-Harvesting Chlorophyll a/b Binding Proteins Lhcb1 and Lhcb2 Play Complementary Roles during State Transitions in Arabidopsis. *Plant Cell* **26**:3646-3660.

Polle, J.E.W., Kanakagiri, S., Jin, E., Masuda, T., and Melis, A. (2002). Truncated chlorophyll antenna size of the photosystems - a practical method to improve microalgal productivity and hydrogen production in mass culture. *Int. J. Hydrogen Energ.* **27**:1257-1264.

Polle, J.E.W., Niyogi, K.K., and Melis, A. (2001). Absence of lutein, violaxanthin and neoxanthin affects the functional chlorophyll antenna size of photosystem-II but not that of photosystem-I in the green alga *Chlamydomonas reinhardtii*. *Plant Cell Physiol.* **42**:482-491.

Popescu, C.E., Borza, T., Bielawski, J.P., and Lee, R.W. (2006). Evolutionary rates and expression level in *Chlamydomonas*. *Genetics* **172**:1567-1576.

Porro, D., Sauer, M., Branduardi, P., and Mattanovich, D. (2005). Recombinant protein production in yeasts. *Mol. Biotechnol.* **31**:245-259.

Pribil, M., Pesaresi, P., Hertle, A., Barbato, R., and Leister, D. (2010). Role of plastid protein phosphatase TAP38 in LHCII dephosphorylation and thylakoid electron flow. *PLoS Biol.* **8**:e1000288.

Punt, P.J., van Biezen, N., Conesa, A., Albers, A., Mangnus, J., and van den Hondel, C. (2002). Filamentous fungi as cell factories for heterologous protein production. *Trends Biotechnol.* **20**:200-206.

Pursiheimo, S., Mulo, P., Rintamaki, E., and Aro, E.M. (2001). Coregulation of light-harvesting complex II phosphorylation and lhcb mRNA accumulation in winter rye. *Plant J.* **26**:317-327.

Puthiyaveetil, S. (2011). A mechanism for regulation of chloroplast LHC II kinase by plastoquinol and thioredoxin. *FEBS Lett.* **585**:1717-1721.

Quinn, J.M., Eriksson, M., Moseley, J.L., and Merchant, S. (2002). Oxygen deficiency responsive gene expression in *Chlamydomonas reinhardtii* through a copper-sensing signal transduction pathway. *Plant. Physiol.* **128**:463-471.

Raghavendra, A.S. and Padmasree, K. (2003). Beneficial interactions of mitochondrial metabolism with photosynthetic carbon assimilation. *Trends Plant Sci.* **8**:546-553.

Ramazanov, Z. and Cardenas, J. (1992). Involvement of photorespiration and glycolate pathway in carbonic anhydrase induction and inorganic carbon concentration in *Chlamydomonas reinhardtii*. *Physiol. Plantarum* **84**:502-508.

Renberg, L., Johansson, A.I., Shutova, T., Stenlund, H., Aksmann, A., Raven, J.A., Gardestrom, P., Moritz, T., and Samuelsson, G. (2010). A metabolomic approach to study major metabolite changes during acclimation to limiting CO_2 in *Chlamydomonas reinhardtii*. *Plant Physiol.* **154**:187-196.

Rintamäki, E., Martinsuo, P., Pursiheimo, S., and Aro, E.M. (2000). Cooperative regulation of light-harvesting complex II phosphorylation via the plastoquinol and ferredoxin-thioredoxin system in chloroplasts. *Proc. Natl. Acad. Sci. USA* **97**:11644–11649.

Rintamäki, E., Salonen, M., Suoranta, U.-M., Carlberg, I., Andersson, B., and Aro, E.-M. (1997). Phosphorylation of light-harvesting complex II and photosystem II core proteins shows different irradiance-dependent regulation *in vivo*: application of phosphothreonine antibodies to analysis of thylakoid phosphoproteins. *J. Biol. Chem.* **272**:30476-30482.

Roach, T., Na, C.S., and Liszkay, A. (2015). High light-induced hydrogen peroxide production in *Chlamydomonas reinhardtii* is increased by high CO_2 availability. *Plant J.* **81**:759-766.

Roach, T., Sedoud, A., and Krieger-Liszkay, A. (2013). Acetate in mixotrophic growth medium affects photosystem II in *Chlamydomonas reinhardtii* and protects against photoinhibition. *BBA-Bioenergetics* **1827**:1183-1190.

Rochaix, J.D. (2013). Redox regulation of thylakoid protein kinases and photosynthetic gene expression. *Antioxid. Redox Sign.* **18**:2184-2201.

Roda, A., Pasini, P., Mirasoli, M., Michelini, E., and Guardigli, M. (2004). Biotechnological applications of bioluminescence and chemiluminescence. *Trends Biotech.* **22**:295-303.

Rosellini, D. (2012). Selectable Markers and Reporter Genes: A Well Furnished Toolbox for Plant Science and Genetic Engineering. *Crit. Rev. Plant Sci.* **31**:401-453.

Rouhier, N., Villarejo, A., Srivastava, M., Gelhaye, E., Keech, O., Droux, M., Finkemeier, I., Samuelsson, G., Dietz, K.J., and Jacquot, J.-P. (2005). Identification of plant glutaredoxin targets. *Antioxid. Redox Sign.* **7**:919-929.

Ruban, A.V. (2015). Evolution under the sun: optimizing light harvesting in photosynthesis. *J. Exp. Bot.* **66**:7-23.

Ruban, A.V., Berera, R., Ilioaia, C., van Stokkum, I.H.M., Kennis, J.T.M., Pascal, A.A., van Amerongen, H., Robert, B., Horton, P., and van Grondelle, R. (2007). Identification of a mechanism of photoprotective energy dissipation in higher plants. *Nature* **450**:575-578.

Ruban, A.V. and Johnson, M.P. (2009). Dynamics of higher plant photosystem cross-section associated with state transitions. *Photosynth. Res.* **99**:173-183.

Ruecker, O., Zillner, K., Groebner-Ferreira, R., and Heitzer, M. (2008). *Gaussia*-luciferase as a sensitive reporter gene for monitoring promoter activity in the nucleus of the green alga *Chlamydomonas reinhardtii*. *Mol. Genet. Genomics* **280**:153-162.

Sahdev, S., Khattar, S.K., and Saini, K.S. (2008). Production of active eukaryotic proteins through bacterial expression systems: a review of the existing biotechnology strategies. *Mol. Cell. Biochem.* **307**:249-264.

Sakihama, Y., Nakamura, S., and Yamasaki, H. (2002). Nitric oxide production mediated by nitrate reductase in the green alga *Chlamydomonas reinhardtii*: an alternative NO production pathway in photosynthetic organisms. *Plant Cell Physiol.* **43**:290-297.

Sarkar, N., Lemaire, S., Wu-Scharf, D., Issakidis-Bourguet, E., and Cerutti, H. (2005). Functional specialization of *Chlamydomonas reinhardtii* cytosolic thioredoxin h1 in the response to alkylation-induced DNA damage. *Eukaryot. Cell* **4**:262-273.

Sawyer, A., Hankamer, B., and Ross, I. (2015). Sulphur responsiveness of the *Chlamydomonas reinhardtii LHCBM9* promoter. *Planta* doi 10.1007/s00425-015-2249-9.

Schägger, H. and von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166**:368-379.

Scheibe, R. (2004). Malate valves to balance cellular energy supply. *Physiol. Plantarum* **120**:21-26.

Scheibe, R. and Dietz, K.-J. (2012). Reduction–oxidation network for flexible adjustment of cellular metabolism in photoautotrophic cells. *Plant Cell Environ.* **35**:202-216.

Scheibe, R. and Jacquot, J.P. (1983). NADP regulates the light activation of NADP-dependent malate dehydrogenase. *Planta* **157**:548-553.

Schmidt, F. (2004). Recombinant expression systems in the pharmaceutical industry. *Appl. Microbiol. Biot.* **65**:363-372.

Schmollinger, S., Mühlhaus, T., Boyle, N.R., Blaby, I.K., Casero, D., Mettler, T., Moseley, J.L., Kropat, J., Sommer, F., Strenkert, D., et al. (2014). Nitrogen-sparing mechanisms in *Chlamydomonas* affect the transcriptome, the proteome, and photosynthetic metabolism. *Plant Cell* **26**:1410-1435.

Schroda, M., Blocker, D., and Beck, C.F. (2000). The HSP70A promoter as a tool for the improved expression of transgenes in *Chlamydomonas*. *Plant J.* **21**:121-131.

Schwarz, C., Bohne, A.V., Wang, F., Cejudo, F.J., and Nickelsen, J. (2012). An intermolecular disulfide-based light switch for chloroplast psbD gene expression in *Chlamydomonas reinhardtii*. *Plant J.* **72**:378-389.

Schwarzländer, M. and Finkemeier, I. (2013). Mitochondrial energy and redox signaling in plants. *Antioxid. redox sign.* **18**:2122-2144.

Scotto-Lavino, E., Du, G., and Frohman, M.A. (2007). Amplification of 5' end cDNA with 'new RACE'. *Nat. Prot.* **1**:3056-3061.

Serrato, A.J., Fernandez-Trijueque, J., Barajas-Lopez, J.D., Chueca, A., and Sahrawy, M. (2013). Plastid thioredoxins: a "one-for-all" redox-signaling system in plants. *Front. Plant Sci.* **4**:Nr. 463

Shao, N. and Bock, R. (2008). A codon-optimized luciferase from *Gaussia princeps* facilitates the in vivo monitoring of gene expression in the model alga *Chlamydomonas reinhardtii*. *Curr. Genet.* **53**:381-388.

Shapiguzov, A., Ingelsson, B., Samol, I., Andres, C., Kessler, F., Rochaix, J.-D., Vener, A.V., and Goldschmidt-Clermont, M. (2010). The PPH1 phosphatase is specifically involved in LHCII dephosphorylation and state transitions in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* **107**:4782-4787.

Shapira, M., Lers, A., Heifetz, P.B., Irihimovitz, V., Osmond, C.B., Gillham, N.W., and Boynton, J.E. (1997). Differential regulation of chloroplast gene expression in *Chlamydomonas reinhardtii* during photoacclimation: light stress transiently suppresses synthesis of the Rubisco LSU protein while enhancing synthesis of the PS II D1 protein. *Plant Mol. Biol.* **33**:1001-1001.

Sherameti, I., Nakamura, M., Yamamoto, Y.Y., Pfannschmidt, T., Obokata, J., and Oelmüller, R. (2002). Polyribosome loading of spinach mRNAs for photosystem I subunits is controlled by photosynthetic electron transport. *Plant J.* **32**:631-639.

Shevela, D., Klimov, V., and Messinger, J. (2007). Interactions of photosystem II with bicarbonate, formate and acetate. *Photosynth. Res.* **94**:247-264.

Sidebottom, C., Buckley, S., Pudney, P., Twigg, S., Jarman, C., Holt, C., Telford, J., McArthur, A., Worrall, D., Hubbard, R., et al. (2000). Phytochemistry: Heat-stable antifreeze protein from grass. *Nature* **406**:256-256.

Sirisha, V.L., Sinha, M., and D'Souza, J.S. (2014). Menadione-induced caspase-dependent programmed cell death in the green chlorophyte *Chlamydomonas reinhardtii*. *J. Phycol.* **50**:587-601.

Slade, W.O., Werth, E.G., Chao, A., and Hicks, L.M. (2014). Phosphoproteomics in photosynthetic organisms. *Electrophoresis* **35**:3441-3451.

Smith, B.M., Morrissey, P.J., Guenther, J.E., Nemson, J.A., Harrison, M.A., Allen, J.F., and Melis, A. (1990). Response of the photosynthetic apparatus in *Dunaliella salina* (green algae) to irradiance stress. *Plant Physiol.* **93**:1433-1440.

Spadaro, D., Yun, B.W., Spoel, S.H., Chu, C.C., Wang, Y.Q., and Loake, G.J. (2010). The redox switch: dynamic regulation of protein function by cysteine modifications. *Physiol. Plantarum* **138**:360-371.

Spalding, M.H. (2009). The CO_2-concentrating mechanism and carbon assimilation. In: The Chlamydomonas Sourcebook: Organellar and metabolic processes. Vol. 2, 2nd ed., --Stern, DB, ed. Academic Press, San Diego. 257-302.

Spalding, M.H., Critchley, C., Govindjee, and Orgren, W.L. (1984). Influence of carbon dioxide concentration during growth on fluorescence induction characteristics of the green alga *Chlamydomonas reinhardtii*. *Photosynth. Res.* **5**:169-176.

Spalding, M.H. and Ogren, W.L. (1982). Photosynthesis is required for induction of the CO_2-concentrating system in *Chlamydomonas reinhardii*. *FEBS Lett.* **145**:41-44.

Specht, E., Miyake-Stoner, S., and Mayfield, S. (2010). Micro-algae come of age as a platform for recombinant protein production. *Biotechnol. Lett.* **32**:1373-1383.

Standfuss, J., Terwisscha van Scheltinga, A.C., Lamborghini, M., and Kühlbrandt, W. (2005). Mechanisms of photoprotection and nonphotochemical quenching in pea light-harvesting complex at 2.5 Å resolution. *EMBO J.* **24**:919-928.

Stengel, A., Benz, J.P., Buchanan, B.B., Soll, J., and Boelter, B. (2009). Preprotein import into chloroplasts via the Toc and Tic complexes is regulated by redox signals in *Pisum sativum*. *Mol. Plant* **2**:1181-1197.

Strenkert, D., Schmollinger, S., and Schroda, M. (2013). Heat shock factor 1 counteracts epigenetic silencing of nuclear transgenes in *Chlamydomonas reinhardtii*. *Nucleic Acids Res.* **41**:5273-5289.

Sueoka, N. (1960). Mitotic replication of deoxyribonucleic acid in *Chlamydomonas reinhardi*. *Proc. Natl. Acad. Sci. USA* **46**:83.

Sun, R., Liu, K., Dong, L., Wu, Y., Paulsen, H., and Yang, C. (2015). Direct energy transfer from the major antenna to the photosystem II core complexes in the absence of minor antennae in liposomes. *BBA-Bioenergetics* **1847**:248-261.

Suzuki, N., Koussevitzky, S., Mittler, R.O.N., and Miller, G.A.D. (2012). ROS and redox signalling in the response of plants to abiotic stress. *Plant Cell Environ.* **35**:259-270.

Szabo, I., Bergantino, E., and Giacometti, G.M. (2005). Light and oxygenic photosynthesis: energy dissipation as a protection mechanism against photo-oxidation. *EMBO Rep.* **6**:629-634.

Tada, Y., Spoel, S.H., Pajerowska-Mukhtar, K., Mou, Z., Song, J., Wang, C., Zuo, J., and Dong, X. (2008). Plant immunity requires conformational charges of NPR1 via S-nitrosylation and thioredoxins. *Science* **321**:952-956.

Takahashi, H., Clowez, S., Wollman, F.-A., Vallon, O., and Rappaport, F. (2013). Cyclic electron flow is redox-controlled but independent of state transition. *Nat. Commun.* **4**:Nr.1954.

Takahashi, H., Iwai, M., Takahashi, Y., and Minagawa, J. (2006). Identification of the mobile light-harvesting complex II polypeptides for state transitions in *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. USA* **103**:477-482.

Takahashi, H., Okamuro, A., Minagawa, J., and Takahashi, Y. (2014). Biochemical characterization of photosystem I-associated light-harvesting complexes I and II isolated from state 2 cells of *Chlamydomonas reinhardtii*. *Plant Cell Physiol.* **55**:1437-1449.

Talabardon, M. and Yang, S.T. (2005). Production of GFP and glucoamylase by recombinant *Aspergillus niger*: Effects of fermentation conditions on fungal morphology and protein secretion. *Biotechnol. Progr.* **21**:1389-1400.

Telfer, A., Allen, J.F., Barber, J., and Bennett, J. (1983). Thylakoid protein phosphorylation during State 1—State 2 transitions in osmotically shocked pea chloroplasts. *BBA-Bioenergetics* **722**:176-181.

Teramoto, H., Ishii, A., Kimura, Y., Hasegawa, K., Nakazawa, S., Nakamura, T., Higashi, S., Watanabe, M., and Ono, T. (2006). Action spectrum for expression of the high intensity light-inducible Lhc-like gene Lhl4 in the green alga *Chlamydomonas reinhardtii*. *Plant Cell Physiol.* **47**:419-425.

Teramoto, H., Itoh, T., and Ono, T.-a. (2004). High-intensity-light-dependent and transient expression of new genes encoding distant relatives of light-harvesting chlorophyll-a/b proteins in *Chlamydomonas reinhardtii*. *Plant Cell. Physiol.* **45**:1221-1232.

Teramoto, H., Nakamori, A., Minagawa, J., and Ono, T.-A. (2002). Light-intensity-dependent expression of Lhc gene family encoding light-harvesting chlorophyll-a/b proteins of photosystem II in *Chlamydomonas reinhardtii*. *Plant Physiol.* **130**:325-333.

Teramoto, H., Ono, T., and Minagawa, J. (2001). Identification of Lhcb gene family encoding the light-harvesting chlorophyll-a/b proteins of photosystem II in *Chlamydomonas reinhardtii*. *Plant Cell Physiol.* **42**:849-856.

Terpe, K. (2006). Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems. *Appl. Microbiol. Biot.* **72**:211-222.

Tikkanen, M. and Aro, E.-M. (2014). Integrative regulatory network of plant thylakoid energy transduction. *Trends Plant Sci.* **19**:10-17.

Tokutsu, R., Iwai, M., and Minagawa, J. (2009). CP29, a monomeric light-harvesting complex II protein, is essential for state transitions in *Chlamydomonas reinhardtii*. *J. Biol. Chem.* **284**:7777-7782.

Tokutsu, R., Kato, N., Bui, K.H., Ishikawa, T., and Minagawa, J. (2012). Revisiting the supramolecular organization of photosystem II in *Chlamydomonas reinhardtii*. *J. Biol. Chem.* **287**:31574-31581.

Tokutsu, R. and Minagawa, J. (2013). Energy-dissipative supercomplex of photosystem II associated with LHCSR3 in *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. USA* **110**:10016-10021.

Trebitsh, T., Levitan, A., Sofer, A., and Danon, A. (2000). Translation of chloroplast psbA mRNA is modulated in the light by counteracting oxidizing and reducing activities. *Mol. Cell. Biol.* **20**:1116-1123.

Trebst, A. (2007). Inhibitors in the functional dissection of the photosynthetic electron transport system. *Photosynth. Res.* **92**:217-224.

Ünlü, C., Drop, B., Croce, R., and van Amerongen, H. (2014). State transitions in *Chlamydomonas reinhardtii* strongly modulate the functional size of photosystem II but not of photosystem I. *Proc. Nat. Acad. Sci. USA* **111**:3460-3465.

Verhaegen, M. and Christopoulos, T.K. (2002). Recombinant *Gaussia* luciferase. Overexpression, purification, and analytical application of a bioluminescent reporter for DNA hybridization. *Anal. Chem.* **74**:4378-4385.

Villarejo, A., Martínez, F., and Ramazanov, Z. (1997). Effect of aminooxyacetate, an inhibitor blocking the glycolate pathway, on the induction of a CO_2-concentrating mechanism and low-CO_2-inducible polypeptides in *Chlamydomonas reinhardtii* (Chlorophyta). *Eur. J. Phycol.* **32**:141-145.

Vogel, M.O., Moore, M., König, K., Pecher, P., Alsharafa, K., Lee, J., and Dietz, K.-J. (2014). Fast retrograde signaling in response to high light involves metabolite export, mitogen-activated protein kinase 6, and AP2/ERF transcription factors in *Arabidopsis*. *Plant Cell* **26**:1151-1165.

von der Heyde, E.L., Klein, B., Abram, L., and Hallmann, A. (2015). The inducible *nitA* promoter provides a powerful molecular switch for transgene expression in *Volvox carteri*. *BMC Biotechnol.* **15**:5.

von Gromoff, E.D., Schroda, M., Oster, U., and Beck, C.F. (2006). Identification of a plastid response element that acts as an enhancer within the *Chlamydomonas* HSP70A promoter. *Nucleic Acids Res.* **34**:4767-4779.

Voß, B., Meinecke, L., Kurz, T., Al-Babili, S., Beck, C.F., and Hess, W.R. (2011). Hemin and magnesium-protoporphyrin IX induce global changes in gene expression in *Chlamydomonas reinhardtii*. *Plant Physiol.* **155**:892-905.

Wang, Y., Duanmu, D., and Spalding, M. (2011). Carbon dioxide concentrating mechanism in *Chlamydomonas reinhardtii*: inorganic carbon transport and CO_2 recapture. *Photosynth. Res.* **109**:115-122.

Wang, Y., Stessman, D.J., and Spalding, M.H. (2015). The CO_2 concentrating mechanism and photosynthetic carbon assimilation in limiting CO_2: how *Chlamydomonas* works against the gradient. *Plant J.* 10.1111/tpj.12829.

Webb, M.R. and Melis, A. (1995). Chloroplast Response in *Dunaliella salina* to Irradiance Stress (Effect on Thylakoid Membrane Protein Assembly and Function). *Plant Physiol.* **107**:885-893.

Wei, L., Derrien, B., Gautier, A., Houille-Vernes, L., Boulouis, A., Saint-Marcoux, D., Malnoë, A., Rappaport, F., de Vitry, C., and Vallon, O. (2014). Nitric oxide-triggered remodeling of chloroplast bioenergetics and thylakoid proteins upon nitrogen starvation in *Chlamydomonas reinhardtii*. *Plant Cell* **26**:353-372.

Weise, A., Altmann, F., Rodriguez-Franco, M., Sjoberg, E.R., Baeumer, W., Launhardt, H., Kietzmann, M., and Gorr, G. (2007). High-level expression of secreted complex glycosylated recombinant human erythropoietin in the *Physcomitrella* Delta-fuc-t Delta-xyl-t mutant. *Plant Biotech. J.* **5**:389-401.

Wessel, D. and Flügge, U.I. (1984). A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem.* **138**:141-143.

Wientjes, E., Drop, B., Kouřil, R., Boekema, E.J., and Croce, R. (2013a). During state 1 to state 2 transition in *Arabidopsis thaliana*, the photosystem II supercomplex gets phosphorylated but does not disassemble. *J. Biol. Chem.* **288**:32821-32826.

Wientjes, E., van Amerongen, H., and Croce, R. (2013b). LHCII is an antenna of both photosystems after long-term acclimation. *BBA-Bioenergetics* **1827**:420-426.

Wiessner, W. (1965). Quantum requirement for acetate assimilation and its significance for quantum measurements in photophosphorylation. *Nature* **205**:56.

Wimalanathan, K. (2011). Genome-wide Computational Analysis of *Chlamydomonas reinhardtii* Promoters. Master Thesis. *Miami University,*

Winck, F.V., Arvidsson, S., Riaño-Pachón, D.M., Hempel, S., Koseska, A., Nikoloski, Z., Urbina Gomez, D.A., Rupprecht, J., and Mueller-Roeber, B. (2013a). Genome-wide identification of regulatory elements and reconstruction of gene regulatory networks of the green alga *Chlamydomonas reinhardtii* under carbon deprivation. *PLoS ONE* **8**:e79909.

Winck, F.V., Páez Melo, D.O., and González Barrios, A.F. (2013b). Carbon acquisition and accumulation in microalgae *Chlamydomonas*: Insights from "omics" approaches. *J. Proteomics* **94**:207-218.

Wingler, A., Lea, P.J., Quick, W.P., and Leegood, R.C. (2000). Photorespiration: metabolic pathways and their role in stress protection. *Phil. Trans. R. Soc. Lond. B* **355**:1517-1529.

Witkin, E.M. (1947). Mutations in *Escherichia coli* induced by chemical agents. *Cold S. H. Q. B.* **12**:256-269.

Wlodarczyk, Lucyna M., Snellenburg, Joris J., Ihalainen, Janne A., van Grondelle, R., van Stokkum, Ivo H.M., and Dekker, Jan P. (2015). Functional rearrangement of the light-harvesting antenna upon state transitions in a green alga. *Biophys. J.* **108**:261-271.

Wobbe, L. (2007). Der cytosolische Translationsrepressor NAB1 in der einzelligen Grünalge *C. reinhardtii*. Dissertation. *Bielefeld University, Faculty of Biology*

Wobbe, L., Blifernez, O., Schwarz, C., Mussgnug, J.H., Nickelsen, J., and Kruse, O. (2009). Cysteine modification of a specific repressor protein controls the translational status of nucleus-encoded LHCII mRNAs in *Chlamydomonas*. *Proc. Natl. Acad. Sci. USA* **106**:13290-13295.

Wobbe, L., Schwarz, C., Nickelsen, J., and Kruse, O. (2008). Translational control of photosynthetic gene expression in phototrophic eukaryotes. *Physiol. Plantarum* **133**:507-515.

Wolfe, G.R., Cunningham, F.X., Durnford, D., Green, B.R., and Gantt, E. (1994). Evidence for a common origin of chloroplasts with light-harvesting complexes of different pigmentation. *Nature* **367**:566-568.

Wollman, F.A. and Delepelaire, P. (1984). Correlation between changes in light energy distribution and changes in thylakoid membrane polypeptide phosphorylation in *Chlamydomonas reinhardtii*. *J. Cell Biol.* **98**:1-7.

Woodson, J.D. and Chory, J. (2008). Coordination of gene expression between organellar and nuclear genomes. *Nat. Rev. Genet.* **9**:383-395.

Wormuth, D., Baier, M., Kandlbinder, A., Scheibe, R., Hartung, W., and Dietz, K.-J. (2006). Regulation of gene expression by photosynthetic signals triggered through modified CO_2 availability. *BMC Plant Biol.* **6**:15.

Wunder, T., Liu, Q., Aseeva, E., Bonardi, V., Leister, D., and Pribil, M. (2012). Control of *STN7* transcript abundance and transient STN7 dimerisation are involved in the regulation of STN7 activity. *Planta* **237**:541-558.

Xiang, Y., Zhang, J., and Weeks, D.P. (2001). The CIA5 gene controls formation of the carbon concentrating mechanism in *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. USA* **98**:5341-5346.

Xie, Y., Mao, Y., Lai, D., Zhang, W., Zheng, T., and Shen, W. (2013). Roles of NIA/NR/NOA1-dependent nitric oxide production and HY1 expression in the modulation of *Arabidopsis* salt tolerance. *J. Exp. Bot.* **64**:3045-3060.

Xue, H., Tokutsu, R., Bergner, S.V., Scholz, M., Minagawa, J., and Hippler, M. (2015). PSBR is required for efficient binding of LHCSR3 to photosystem II - light-harvesting supercomplexes in *Chlamydomonas reinhardtii*. *Plant Physiol.* **167**:1566-1578.

Yamamoto, H.Y., Chichester, C.O., and Nakayama, T.O. (1962). Studies on light and dark interconversions of leaf xanthophylls. *Arch. Biochem. Biophys.* **97**:168-173.

Yamamoto, Y.Y., Ichida, H., Matsui, M., Obokata, J., Sakurai, T., Satou, M., Seki, M., Shinozaki, K., and Abe, T. (2007). Identification of plant promoter constituents by analysis of local distribution of short sequences. *BMC genomics* **8**:67.

Yamano, T., Miura, K., and Fukuzawa, H. (2008). Expression analysis of genes associated with the induction of the carbon-concentrating mechanism in *Chlamydomonas reinhardtii*. *Plant Physiol.* **147**:340-354.

Yamazaki, D., Motohashi, K., Kasama, T., Hara, Y., and Hisabori, T. (2004). Target proteins of the cytosolic thioredoxins in *Arabidopsis thaliana*. *Plant Cell Physiol.* **45**:18-27.

Yang, C., Bolotin, E., Jiang, T., Sladek, F.M., and Martinez, E. (2007). Prevalence of the initiator over the TATA box in human and yeast genes and identification of DNA motifs enriched in human TATA-less core promoters. *Gene* **389**:52.

Yoshioka, S., Taniguchi, F., Miura, K., Inoue, T., Yamano, T., and Fukuzawa, H. (2004). The novel Myb transcription factor LCR1 regulates the CO_2-responsive gene CAH1, encoding a periplasmic carbonic anhydrase in *Chlamydomonas reinhardtii*. *Plant Cell* **16**:1466-1477.

Yu, J. and Langridge, W. (2003). Expression of rotavirus capsid protein VP6 in transgenic potato and its oral immunogenicity in mice. *Transgenic Res.* **12**:163-169.

Zaffagnini, M., Bedhomme, M., Marchand, C.H., Morisse, S., Trost, P., and Lemaire, S.D. (2012). Redox regulation in photosynthetic organisms: Focus on glutathionylation. *Antioxid. Redox Sign.* **16**:567-586.

Zaffagnini, M., Michelet, L., Marchand, C., Sparla, F., Decottignies, P., Le Marechal, P., Miginiac-Maslow, M., Noctor, G., Trost, P., and Lemaire, S.D. (2007). The thioredoxin-independent isoform of chloroplastic glyceraldehyde-3-phosphate dehydrogenase is selectively regulated by glutathionylation. *FEBS J.* **274**:212-226.

Zaffagnini, M., Morisse, S., Bedhomme, M., Marchand, C.H., Festa, M., Rouhier, N., Lemaire, S.D., and Trost, P. (2013). Mechanisms of nitrosylation and denitrosylation of cytoplasmic glyceraldehyde-3-phosphate dehydrogenase from *Arabidopsis thaliana*. *J. Biol. Chem.* **288**:22777-22789.

Zhang, X.-W., Chen, F., and Johns, M.R. (1999). Kinetic models for heterotrophic growth of *Chlamydomonas reinhardtii* in batch and fed-batch cultures. *Process Biochem.* **35**:385-389.

Zhou, D.X. (1999). Regulatory mechanism of plant gene transcription by GT-elements and GT-factors. *Trends Plant Sci.* **4**:210-214.

Zito, F., Finazzi, G., Delosme, R., Nitschke, W., Picot, D., and Wollman, F.A. (1999). The Qo site of cytochrome b6f complexes controls the activation of the LHCII kinase. *EMBO J.* **18**:2961-2969.

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PUBLIKATIONEN

Berger, H., de Mia, M., Morisse, S., Marchand, C., Lemaire, S., Wobbe, L., and Kruse, O. (2015). A light switch based on protein S-nitrosylation fine-tunes photosynthetic light-harvesting in the microalga *Chlamydomonas reinhardtii*. *In preparation for submission*

Berger, H., Blifernez-Klassen, O., Ballottari, M., Bassi, R., Wobbe, L. and Kruse, O. (2014). Integration of carbon assimilation modes with photosynthetic light capture in the green alga *Chlamydomonas reinhardtii*. *Molecular Plant* 7(10), 1545-1559

Lauersen, K.J., Vanderveer, T.L., **Berger, H.**, Kaluza, I., Mussgnug, J.H., Walker, V.K. and Kruse, O. (2013). Ice recrystallization inhibition mediated by a nuclear-expressed and -secreted recombinant ice-binding protein in the microalga *Chlamydomonas reinhardtii*. *Applied Microbiology and Biotechnology* 97(22), 9763-9772.

Lauersen, K.J., **Berger, H.**, Mussgnug, J.H. and Kruse, O. (2013). Efficient recombinant protein production and secretion from nuclear transgenes in *Chlamydomonas reinhardtii*. *Journal of Biotechnology* 167(2), 101–110.

Danksagung

An dieser Stelle möchte ich mich recht herzlich bei all jenen bedanken, die mich im Laufe meiner Promotion bei der Forschung und Erstellung der Dissertation begleitet und unterstützt haben. Folgenden Personen möchte ich meinen besonderen Dank aussprechen:

Prof. Dr. Olaf Kruse für die Möglichkeit meine Doktorarbeit am Lehrstuhl Algenbiotechnologie und Bioenergie der Universität Bielefeld anfertigen zu dürfen, die interessante Aufgabenstellung, die exzellente Betreuung und die stete Unterstützung,

Prof. Dr. Harald Paulsen für die freundliche Übernahme des Zweitgutachtens,

Dr. Lutz Wobbe für die vielen hilfreichen Diskussionen, Tipps und Korrekturen,

meinen Kooperationspartnern Dr. Stephane Lemaire, Dr. Samuel Morisse, Dr. Christophe Marchand und Marcello de Mia des CNRS/UPMC Paris sowie Prof. Dr. Roberto Bassi und apl. Prof. Dr. Matteo Ballottari der Universität Verona für die fruchtbare Zusammenarbeit,

der gesamten Arbeitsgruppe für das stets angenehme Klima innerhalb und außerhalb des Labors; insbesondere Olga für den kreativen und ermunternden Austausch, Viktor für den die Kreativität beflügelnden Kaffeenachschub, Robin für das Korrigieren vieler Texte, Kristin für die Algennothilfe, Daniel für schöne Worte, Louise und Tatjana für die fleißige Unterstützung beim Promoterprojekt, Kyle für das american r, Anja für ihre Energie, Jan für Autogrammstunden, Lisa und Sabrina für die nette Büroatmosphäre, Andreas für tolle Kuchen, Christian für philosophische Exkurse, und, last not least, Susanne für die Entwirrung von Formalitäten; sowie weiteren Mitgliedern der Fakultät für Biologie und des CeBiTec für ihre Hilfsbereitschaft,

der Deutschen Forschungsgemeinschaft für die Bereitstellung finanzieller Mittel,

dem Rektorat der Universität Bielefeld für die Folgefinanzierung,

sowie Adi für die vielen geduldsamen Hilfestellungen und Sarah für den Blick mit Abstand.

Besonderer Dank gilt meiner Familie und meinen Freunden für den liebevollen Rückhalt.

Erklärung

Hiermit versichere ich, dass ich die vorliegende Dissertation selbständig angefertigt und dazu keine anderen als die angegebenen Hilfsmittel und Quellen verwendet habe.

Teile dieser Arbeit wurden veröffentlicht, wie an den entsprechenden Stellen kenntlich gemacht.

Weiterhin erkläre ich, dass die vorliegende Schrift weder vollständig noch teilweise einer anderen Fakultät zur Erlangung eines akademischen Grades vorgelegt worden ist. Ich bewerbe mich erstmals um den Doktorgrad der Naturwissenschaften der Universität Bielefeld.

Bielefeld, 13. April 2015 _____

Hanna Berger