Analysis of factors involved in plastid gene expression in the unicellular green alga *Chlamydomonas reinhardtii*

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Christian Schwarz

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Erstgutachter: Prof. Dr. Jörg Nickelsen, AG Molekulare Pflanzenwissenschaften

Zweitgutachter: Prof. Dr. Peter Geigenberger, AG Pflanzenmetabolismus

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Abbreviations

ADP	adenosine diphosphate
ATP	adenosine triphosphate
CDJ	chloroplast DnaJ-like
CES	control of epistasy by synthesis
CP	chlorophyll binding protein
CRP	chloroplast RNA processing
DNA	deoxyribonucleic acid
dsRNA	double-stranded ribonucleic acid
ER	endoplasmic reticulum
FAD	flavin adenine dinucleotide
HMW	high molecular weight
HMWC	high molecular weight complex
hnRNP K	heterogeneous nuclear ribonucleoprotein K
KH	hnRNP K homology
LDM	low density membrane
LHCII	light harvesting complex of photosystem II
mRNA	messenger RNA
NADP	oxidized form of nicotinamide adenine dinucleotide phosphate
NADPH	reduced form of nicotinamide adenine dinucleotide phosphate
NEP	nucleus encoded plastid RNA polymerase
OPR	octatricopeptide repeat
ORF	open reading frame

PEP	plastid encoded plastid RNA polymerase
pH	negative decimal logarithm of proton activity
PPR	pentatricopeptide repeat
PSI	photosystem I
PSII	photosystem II
PTM	post-translational modification
RB / RBP	RNA binding protein
RBD	RNA binding domain
RNA	ribonucleic acid
RNase	ribonuclease
RNP	ribonucleoprotein
ROS	reactive oxygen species
RRM	RNA recognition motif
rRNA	ribosomal RNA
Rubisco	ribulose-1,5-biphosphat-carboxylase/oxygenase
S	Svedberg, sedimentation coefficient
SD	Shine-Dalgarno
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
ssDNA	single-stranded DNA
TPR	tetratricopeptide repeat
tRNA	transfer RNA
UV	ultraviolet

Gene abbreviations

atpA	gene encoding the α subunit of the CF_1ATP synthase
atpB	gene encoding the β subunit of the CF_1ATP synthase
atpC	gene encoding the γ subunit of the CF_1ATP synthase
lhcb	gene encoding a light harvesting chlorophyll a/b binding protein of photosystem II
lhcbm6	gene encoding the LHCB isoform M6
ndhD	gene encoding the subunit 4 of the NADH dehydrogenase
petA	gene encoding the cytochrome f subunit of the cytochrome b_6f complex
petD	gene encoding the subunit IV of the cytochrome b_6f complex
petE	gene encoding plastocyanin
psaA	gene encoding the P700 apoprotein A1 of PSI
psaB	gene encoding the P700 apoprotein B1 of PSI
psaC	gene encoding the subunit VII of PSI
psbA	gene encoding the D1 protein of PSII
psbB	gene encoding the CP47 protein of PSII
psbC	gene encoding the CP43 protein of PSII
psbD	gene encoding the D2 protein of PSII
psbH	gene encoding the PsbH protein of PSII
rbcL	gene encoding the large subunit of Rubisco
rbcS	gene encoding the small subunit of Rubisco
tscA	gene encoding a chloroplast RNA in *Chlamydomonas reinhardtii* involved in *psaA* splicing

Zusammenfassung

Die Zusammensetzung und Struktur essentieller Proteinkomplexe innerhalb der Chloroplasten, wie die der Photosysteme, des Cytochrom b_6f-Komplexes, der ATP-Synthase und der Ribulose-1,5-bisphosphat-Carboxylase/Oxygenase (Rubisco), sind sehr gut erforscht. Weitaus weniger ist dagegen über die Identität und Funktion von Faktoren, die in die Synthese sowie die Assemblierung der Untereinheiten dieser Komplexe involviert sind, bekannt. Der Schwerpunkt dieser Dissertation lag in der Untersuchung von Proteinen, die auf posttranskriptioneller Ebene regulativ auf die Synthese von Untereinheiten des Photosystems II und der Rubisco einwirken.

Zahlreiche Untersuchungen zur plastidären Genexpression in höheren Pflanzen und Grünalgen belegen die Beteiligung kernkodierter Proteinfaktoren an der Prozessierung und Stabilisierung entsprechender mRNAs sowie an deren Translation. In der hier untersuchten Grünalge *Chlamydomonas reinhardtii* wird die *psbD*-mRNA, welche für das D2-Protein im Reaktionszentrum des Photosystems II (PSII) kodiert, durch Interaktion mit dem kernkodierten Nac2-Protein vor exonukleolytischem Abbau geschützt. Ein weiterer, in die Translation der *psbD*-mRNA involvierter Proteinfaktor RBP40, wurde im Rahmen dieser Arbeit näher charakterisiert. Durch Anwendung verschiedener biochemischer und molekularbiologischer Methoden konnte gezeigt werden, dass Nac2 und RBP40 einen Komplex bilden, wobei RBP40 in Abhängigkeit der Anwesenheit von Nac2 spezifisch an einen poly(U)-Bereich der 5`UTR der *psbD*-mRNA bindet. Es konnte zudem nachgewiesen werden, dass RBP40 zwar mit ribosomaler RNA interagiert, jedoch nicht mehr an *psbD*-RNA gebunden ist, die mit translatierenden Polysomen assoziiert ist, wodurch Rückschlüsse auf die zeitliche Abfolge der D2-Synthese gezogen werden können. Weitere Untersuchungen zeigten eine lichtabhängige, redoxregulierte Assoziation von RBP40 mit dem Nac2-Komplex, die für die Bindung von RBP40 an seine RNA-Zielsequenz sowie der wahrscheinlich daraus resultierenden Auflösung einer Haarnadelstruktur um das *psbD*-Startkodon verantwortlich ist. Dieser Prozess stellt somit einen entscheidenden Kontrollpunkt für die D2-Synthese dar. Die Analyse verschiedener *psbD*-Mutanten, in denen zum einem durch Punktmutationen diese Haarnadelstruktur aufgelöst und zum anderen die RBP40-Bindestelle deletiert wurde, zeigten eine reduzierte, lichtabhängige Steigerung der D2-Synthese. Somit scheint dieser von RBP40 ausgehende Prozess für die Syntheserate von D2 im Licht verantwortlich zu sein. Erste Schritte der *psbD*-Translation beinhalten daher wahrscheinlich eine redoxabhängige Bindung des Nac2/RBP40-Komplexes, wodurch es zur RBP40-vermittelten Auflösung der

Haarnadelstruktur im 5'UTR der *psbD*-mRNA kommt. Die Entfernung dieser RNA-Sekundärstruktur ermöglicht nachfolgend den ribosomalen Zugang zum Translationsinitiationsort.

Darüber hinaus wurde im Rahmen dieser Arbeit ein Translationsrepressor, das Protein NAB1, auf seine mögliche redoxabhängige RNA-Bindungsfähigkeit hin untersucht. NAB1, ein Protein, welches durch Bindung an die *lhcb*-mRNA die Synthese der kodierten Lichtsammelkomplexproteine inhibiert, zeigte hierbei *in vitro* ebenfalls eine vom Redoxzustand bestimmte Interaktion mit der Ziel-RNA.

Der Redoxzustand könnte ebenfalls für Bindung von RNA an Komplexe verantwortlich sein, welche die Eisen-Schwefel-Cluster-haltigen Proteine CDJ3 und CDJ4 aufweisen. Von beiden Proteinen wird angenommen, dass sie redoxabhängig die Spezifität des HSP70B-Chaperonsystems und somit die Organisation regulatorischer Proteinkomplexe beeinflussen. Tatsächlich erwies sich das Protein CDJ3 als Komponente eines hochmolekularen RNA-bindenden Komplexes, was auf eine Chaperon-vermittelte Remodellierung dieses Protein-RNA-Komplexes hinweist. CDJ3 könnte auf diese Weise an der Expression plastidärer Transkripte beteiligt sein. Allerdings scheint es sich hier nicht um einen generellen RNA-bindenden Komplex zu handeln, da beispielsweise keine Interaktion mit *psbD*-mRNA nachgewiesen werden konnte.

Die Regulation der plastidären Genexpression über hochmolekulare RNA-bindende Komplexe scheint einen häufig auftretenden Mechanismus darzustellen. So konnte ebenfalls für das konservierte, kernkodierte Regulatorprotein Mrl1, das sowohl in *Arabidopsis* als auch in *Chlamydomonas* an der Prozessierung der Transkripte der großen Untereinheit der Rubisco (*rbcL*) beteiligt ist, nachgewiesen werden, dass es sich hierbei um eine Komponente eines hochmolekularen *rbcL*-mRNA-bindenden Komplexes handelt. Die im Rahmen dieser Arbeit erlangten Resultate erlauben somit tiefere Einblicke in Mechanismen der plastidären Genexpression sowie in die Organisation involvierter regulativ wirkender RNA-Protein-Komplexe.

Summary

Composition and structure of the essential complexes in the chloroplast, like photosystems, cytochrome b_6f complex, ATP synthase and Rubisco, is well known. Less understood is the regulation of subunit synthesis and assembly into these complexes. The focus of this thesis was the analysis of proteins involved in post-transcriptional regulation of subunit synthesis of PSII and Rubisco.

Several studies about plastid gene expression in higher plants and green algae have proven the participation of nucleus-encoded protein factors in processing and stabilization as well as translation of corresponding mRNAs. One example in the green alga *C. reinhardtii* is the *psbD* mRNA, encoding for the D2 protein of the PSII reaction center. The transcript is protected from exonucleolytic degradation by interacting with the nucleus-encoded Nac2 protein. The additional factor RBP40 which is involved in translation of the same mRNA was investigated in more detail in the framework of this thesis. Interaction between RBP40 and the transcript is mediated by a poly(U)-sequence of the *psbD* 5'UTR. By combining several methods from the fields of biochemistry and molecular biology it was proven that RBP40 binds specifically to the 5`UTR of the *psbD* mRNA in a Nac2 dependent manner. Additionally, it could be shown that, even though RBP40 is binding to ribosomal RNA, it seems not to be associated with polysomes which actively translate *psbD* mRNA. This allows conclusions about the chronology of D2 synthesis. Further investigations established a light-dependent and redox-regulated association of RBP40 to the Nac2 complex, which is involved in the association of RBP40 to its target RNA and subsequent synthesis of the D2 protein. The analysis of *psbD* mutants which exhibit deletion of the RBP40 binding region and point mutations within a hairpin structure downstream of the RBP40 binding site showed different increases in the translation of the *psbD* mRNA when switching from dark to light conditions. Therefore, elevated D2 synthesis rates in the light seem to be determined by a process that involves RBP40. Thus, first steps for translation of the *psbD* RNA likely include a redox-dependent binding of RBP40 to the Nac2 complex and the *psbD* mRNA.

In addition another factor involved in translation, the NAB1 protein, was analyzed within this thesis in regard to its potentially redox-dependent RNA binding ability. Hereby, NAB1, which - by binding to *lhcb* mRNAs - represses the synthesis of light harvesting complex proteins similarly to RBP40 showed a redox-dependent RNA binding activity *in vitro*.

The oxidation level could also be responsible for binding of RNA to complexes containing the iron-sulfur-cluster proteins CDJ3 and CDJ4. It is assumed that both proteins regulate the specificity of HSP70B chaperones in a redox dependent manner and, therefore, the organization of regulatory protein complexes. CDJ3 is part of a high molecular weight RNA-binding complex, suggesting a chaperone-assisted remodeling of this RNA-protein complex and a role for CDJ3 in expression of plastid transcripts. However, this complex does not seem to have a general function since no specific interaction with the *psbD* RNA was shown.

Regulation of chloroplast gene expression by high molecular weight RNA binding complexes seem to be a frequent mechanism. Accordingly, the conserved nucleus-encoded protein MRL1, which is necessary for processing of the transcripts of the large subunit of Rubisco (*rbcL*) in *Arabidopsis* and *Chlamydomonas*, was shown to be a component of a high molecular weight complex binding to *rbcL* mRNA. Therefore, results obtained in this thesis improved the understanding of mechanisms of plastid gene expression as well as the organization of involved regulatory RNA protein complexes.

1. Introduction

Eukaryotic cells of animals and fungi originated from the fusion of an archaebacterium with an α-proteobacterium (Cavalier-Smith 2002). Recent results propose that a likely host of this event belongs to the archaebacterial group of the *Thermoplasmatales* (Pisani, *et al.* 2007). The endosymbiotic proteobacteria evolved to mitochondria (Gray, *et al.* 1999). An additional fusion event with an oxygen-producing cyanobacterium gave rise to the first photoautotroph eukaryotes with chloroplasts (Palmer 1993, Gray 1999, Gould, *et al.* 2008).

1.1 Evolution of organelles: chloroplasts and mitochondria

The evolution of mitochondria definitely enhanced abilities of the host cell to conduct oxidative phosphorylation. This resulted in higher ATP yields, giving an energetic advantage over the "organelle-free" prokaryote that was only able to produce ATP by glycolysis (Cavalier-Smith 2002).

Chloroplasts enable the conversion of light energy into chemical energy as well as storage of carbon as high energy organic compounds, therefore giving their host cells even more metabolic advantages (Gould, *et al.* 2008, Martin 2010).

Since these endosymbiotic events, an ongoing transfer of organellar genes into the nuclear genome can be observed, while some genes that were necessary for the survival of the former independent endosymbiont have been lost (Martin, *et al.* 2002, Raven and Allen 2003, Timmis, *et al.* 2004, Kleine, *et al.* 2009). Whereas chloroplasts and mitochondria still contain their own DNA, the transfer of genes to the nucleus reduced their number in chloroplasts to ~20-200 and 3-63 genes in mitochondria. Approximately 4500 genes of the ancestral endosymbiont are nowadays encoded in the nucleus (Martin, *et al.* 2002, Timmis, *et al.* 2004). Gene transfer does not occur unilaterally in direction to the nucleus. There are also examples of mitochondrial genes being transferred to the chloroplast or putative former nuclear genes which are expressed in mitochondria (Schuster and Brennicke 1987, Brouard, *et al.* 2008). It is also known that proteins which are encoded by genes of cyanobacterial origin now have a function outside of the chloroplast (Vesteg, *et al.* 2009). Some organelle-like mitosomes which are found in various unicellular eukaryotic organisms have even lost all of their genes (Tovar, *et al.* 2003, Martin and Müller 2007). Reasons might be that organellar genomes reproduce asexually. Thus, plastid and mitochondrial genes have higher mutation rates and

selective pressures in comparison to the nuclear genome (reviewed in Maier, *et al.* 2008, Schmitz-Linneweber and Small 2008).

1.2 Photosynthesis

Photosystem I (PSI) and PSII are the most prominent members of the multi-subunit complexes in the thylakoid membranes of the chloroplast. These photosystems, together with an ATP synthase and a cytochrome b_6f complex, enable respective organisms to perform oxygenic photosynthesis, i.e. conversion of sunlight into chemical energy as shown in figure 1.

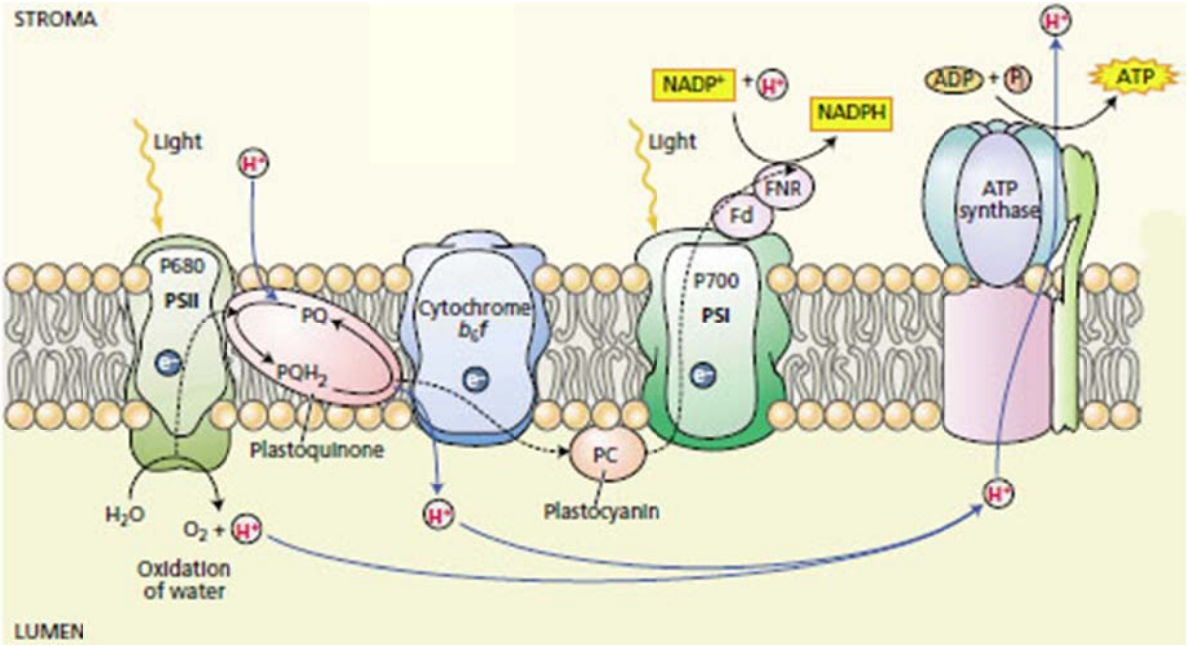

Figure 1: Photosynthesis in the chloroplast (derived from Taiz and Zeiger 2002). Major complexes of the chloroplast and chemical reactions are depicted in a simplified scheme (P680 – PSII primary donor, P700 – PSI primary donor, Fd – ferredoxin, FNR – ferredoxin $NADP^+$ reductase).

PSII absorbs light energy to generate electrons by the oxidation of water to molecular oxygen. Electron transport through plastoquinone, the cytochrome b_6f complex and plastocyanin is used with additional light absorbing reactions in PSI to produce NADPH. The resulting proton gradient of these processes is then used by the ATP synthase to generate ATP, which itself is necessary in the non-light driven reactions of metabolism for the production of carbohydrates by fixation of carbon dioxide (Xiong and Bauer 2002, Nelson and Yocum

2006). The most basic way of describing photosynthesis is the conversion of water, carbon dioxide and light energy to sugars and, as a by-product, molecular oxygen.

Therefore, due to creation of oxygen as its secondary product, oxygenic photosynthesis is responsible for the oxygen content in the atmosphere. Another implication is the constitution of an ozone layer, protecting life forms from harmful levels of UV radiation. These effects allowed for respiration and evolution of all eukaryotes (Blankenship 1992). Photosynthetic reactions can be declared by these arguments to be the most import series of chemical reactions for development of higher life forms (Xiong and Bauer 2002).

As the photosynthetic reactions start with the splitting of water in PSII - a central event of this process - this multimeric complex as it is defined in eukaryotes will be described in more detail. A schematic depiction of the subunit-composition of PSII is shown in figure 2. Water-splitting and binding of several pigments takes place at the reaction center, consisting of the proteins D1 and D2 (Dekker and Boekema 2005). The center is embedded between the chlorophyll-binding proteins CP47 and CP43, representing the inner antenna of the PSII core. Additional intrinsic subunits are assembled around the core. Most of them seem to be involved in assembly, stabilization and dimerization of the PSII complex; their exact number is organism-dependent (Dekker and Boekema 2005). The extrinsic subunits PsbO, PsbP and PsbQ which are positioned on the luminal side constitute the oxygen-evolving complex of PSII (Dekker and Boekema 2005). This monomeric form of PSII undergoes a dimerization event (Nield*, et al.* 2000). Between two and four LHCII (light harvesting complex of PSII) trimers are then attached to the PSII core dimer as peripheral antennae to enable more efficient capture of excitation energy from photons. CP24, CP26 and CP29 serve as linkers for the outer antennae in these supercomplexes (Ruffle and Sayre 1998, Dekker and Boekema 2005).

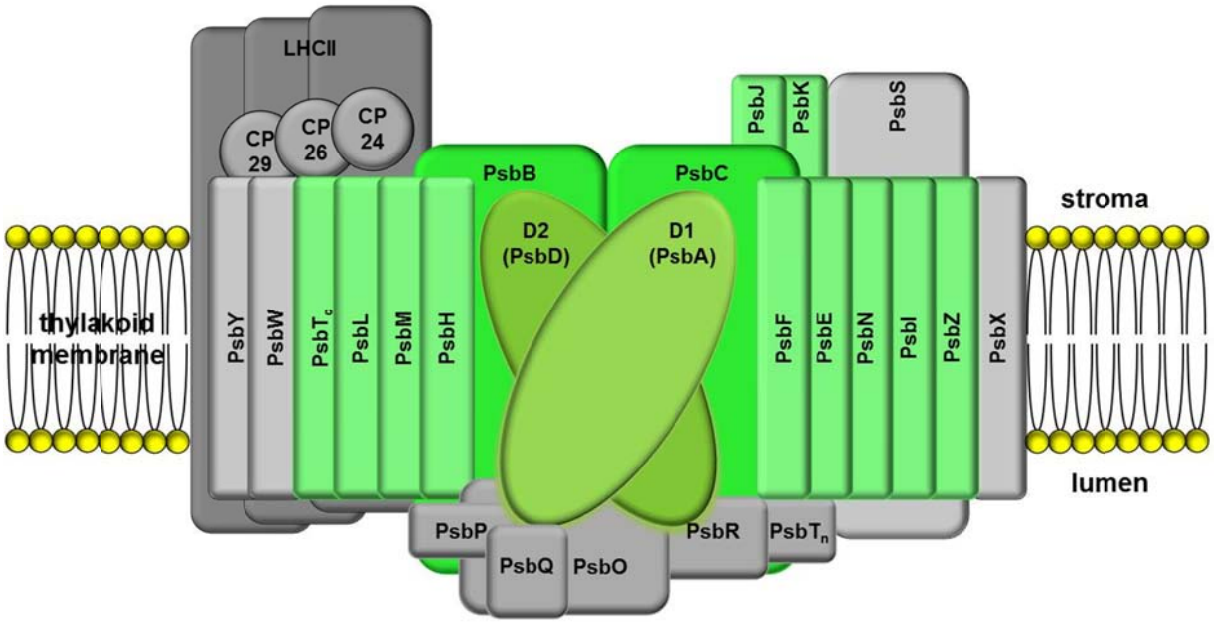

Figure 2: Scheme of protein subunits of the PSII-LHCII complex in plants (derived from Barber, *et al.* 1997, Ruffle and Sayre 1998, Dekker and Boekema 2005, Grossman, *et al.* 2010). Chloroplast encoded subunits are given in green, while nucleus encoded subunits are depicted in grey.

So called "dark reactions" of photosynthesis, as they require no light, are initiated by Rubisco, which adds of carbon dioxide to ribulose-1,5-bisphosphate. The instable product of this addition then dissociates into two molecules of 3-phosphoglycerate (3PG). One 3PG molecule can be used for metabolism. The other 3PG is required for the subsequent regeneration of ribulose-1,5-bisphosphate in several steps - where NADPH and ATP synthesized during photosynthesis are necessary (Bassham, *et al.* 1950). Rubisco also adds oxygen to ribulose-1,5-bisphosphate. This side reaction requires the energy-consuming steps of photorespiration to restore the original substrate for carbon dioxide fixation (Peterhansel and Maurino 2011).

1.3 The model organism *Chlamydomonas reinhardtii*

Of great importance are organisms which facilitate the investigation of above described photosynthetic events. Depending on specific demands, organisms studied include cyanobacteria, algae, mosses, and higher plants. An outstanding model organism for research of chloroplast biogenesis and photosynthesis is the unicellular green alga *C. reinhardtii* because it can grow heterotrophically in acetate-containing media, enabling the analysis of photosynthesis-deficient mutants (Nickelsen and Kück 2000, Harris 2009). Cells as depicted

schematically in figure 3 are about 10 µm in size and contain two isoform flagella. Additional specialties are exactly one cup-shaped chloroplast per cell that contains the photoreceptive "eye spot", which allows *Chlamydomonas* to perform phototaxis and the pyrenoid, a region supposed to be important for carbon dioxide fixation and targeted protein synthesis including assembly of PSII (Uniacke and Zerges 2007, Harris 2009, Uniacke and Zerges 2009). Other important fields of interest investigated in *Chlamydomonas* imply the structure and function of flagella, genetics of basal bodies (centrioles), light perception, cell-cell recognition, and cell cycle control (Harris 2001).

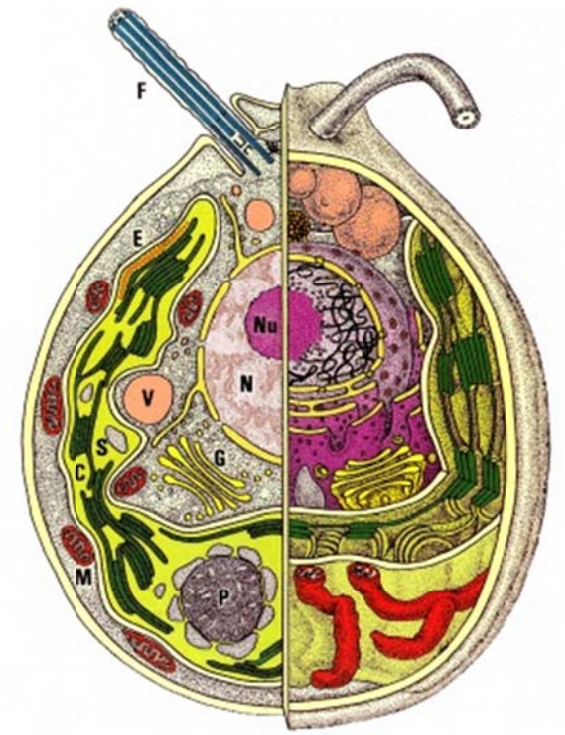

Figure 3: Cross-section scheme of a *C. reinhardtii* cell (Nickelsen and Kück 2000). (C) chloroplast, (E) eye-spot, (F) flagella, (G) Golgi vesicle, (M) mitochondria, (N) nucleus, (Nu) nucleolus, (P) pyrenoid, (S) starch grain, (V) vacuole

Besides being able to assemble complete photosystems in darkness (Malnoë, *et al.* 1988), *Chlamydomonas* exhibits advantages compared to vascular plants including that:

1. Until now, *Chlamydomonas* is the only photoautotrophic eukaryote in which DNA can be readily transformed into the genomes of the nucleus, the chloroplast and mitochondria, enabling specific genetic modifications in all compartments (Boynton, *et al.* 1988, Kindle 1990, Remacle, *et al.* 2006).

2. Its flagella turn *Chlamydomonas* into a promising organism for basic research on human diseases that are caused by cilia/flagella dysfunction, e.g. primary cilia dyskinesia (Snell, *et al.* 2004, Morillas, *et al.* 2007, Pan 2008).
3. It contains both animal- and plant-like genes and can be used to analyze the eukaryotic circadian clock at different levels of organization as the complete genomic sequence and several sub-proteomes are known (Schulze, *et al.* 2010, Matsuo and Ishiura 2011).
4. It can be grown in large numbers at short generation times in low cost media, resulting in high yields of compounds of interest. In case of biofuel production, it does not compete with food crops for arable land. Another example is the overexpression of important proteins like vaccines in a plant system, circumventing problems of animal-based approaches (Rupprecht 2009, Cardi, *et al.* 2010, Schmidt, *et al.* 2010, Specht, *et al.* 2010, Maliga and Bock 2011).

Therefore, *C. reinhardtii* has become an important and frequent tool in biology and biotechnology after being first described by Dangeard in 1888.

1.4 Gene expression in chloroplasts

Non-locally encoded proteins of the chloroplast have to be imported into the organelle after their synthesis in the cytoplasm. Therefore, a highly coordinated expression of nuclear and organellar genomes is an essential prerequisite for proper timely and spatial arrangement of all subunits by the involvement of nucleus-encoded regulatory factors (figure 4; Goldschmidt-Clermont 1998, Barkan and Goldschmidt-Clermont 2000, Woodson and Chory 2008). Even most complex functions in the organelle can be regulated at several levels by this delicate machinery in coordination with signaling pathways, which allows the cell to respond to different kinds of environmental changes by fine-tuning its metabolism (Gray, *et al.* 2003). Changes in cell development that depend on exact stoichiometry of differentially encoded components rely on anterograde signaling, e.g. signals are going from the nucleus to the organelles. Examples are nucleus encoded proteins that determine the rate of existing RNAs in chloroplasts, like Mbb1 in *Chlamydomonas* and its orthologue HCF107 in *Arabidopsis*, which detect *psbB* or *psbH* transcripts, respectively (Vaistij, *et al.* 2000b, Sane, *et al.* 2005). Rapidly changing conditions, i.e. redox levels / oxidative stress and availability of nutrients, have to be reported from the organelles to the nucleus by the process of retrograde signaling as the former endosymbionts have lost their own regulatory mechanisms (Woodson and Chory 2008). This holds true amongst other examples for regulation of chlorophyll

biosynthesis as well as avoidance of ROS (Tanaka and Tanaka 2007, Møller and Sweetlove 2010, Suzuki, *et al.* 2011). A consequence of retrograde signaling is for example the reduction of transcript levels of the nuclear encoded *lhcb1*, *rbcS*, *petE* and *atpC* genes after application of inhibitors of chloroplast translation (Sullivan and Gray 1999).

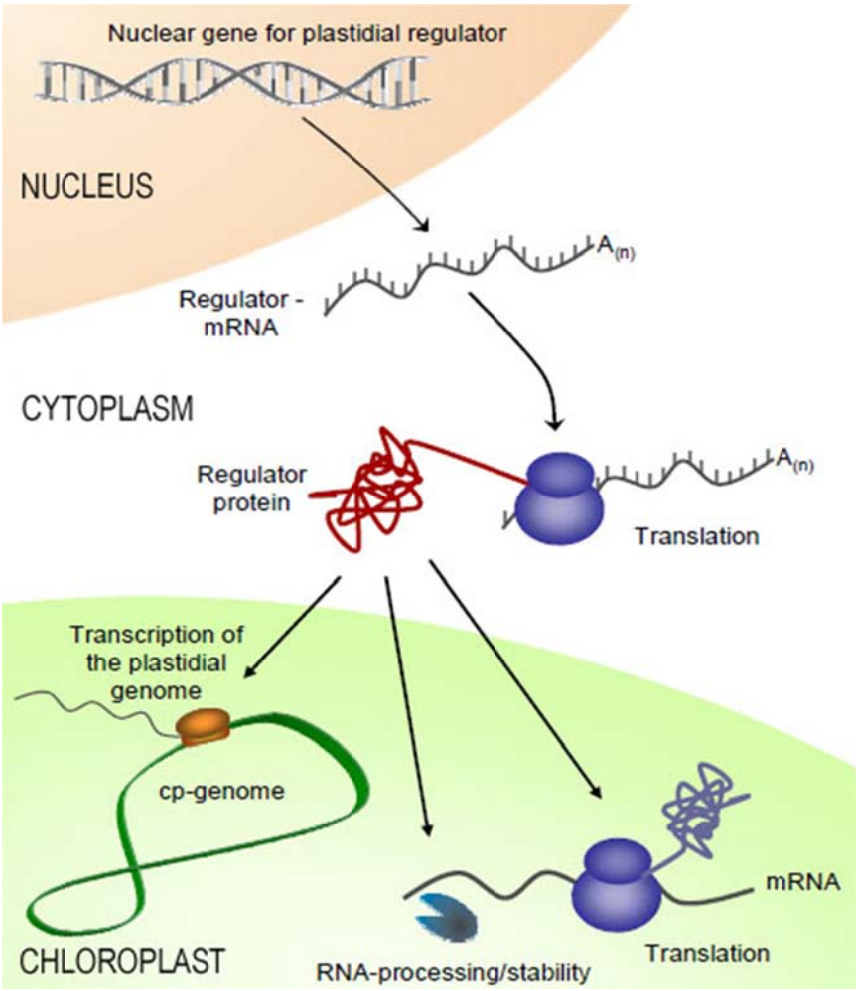

Figure 4: Regulation of chloroplast gene expression via nucleus-encoded regulatory factors (taken from Bohne, *et al.* 2009). Expression of plastid genes is regulated at the transcriptional, post-transcriptional and translational level of synthesis by regulatory proteins encoded in the nucleus.

Another important part of intracellular communication is represented by light-signaling pathways (reviewed in Chory 2010). The perception of different light qualities is mediated by photoreceptors. Blue-light responding cryptochromes are supposed to influence early blue-light induced genes, whose gene products lead to irreversible changes like expansion and opening of cotyledons (Ohgishi, *et al.* 2004). Phototropins, first identified in *Arabidopsis* in 1997, like light activated kinases also respond to blue light and mainly function in

transcriptional regulation to optimize processes involved in photosynthesis (Huala, *et al.* 1997, reviewed in Christie 2007, Goh 2009). Receptors for red and far-red wavelengths include the phytochromes and phytochrome-related sensors which are present amongst others in unicellular green algae, diatoms, cyanobacteria, non-oxygenic photosynthetic bacteria, non-photosynthetic bacteria, and even fungi. The first red light receptor in plants was already characterized at the molecular level in 1984 (Hershey, *et al.* 1984, reviewed in Rockwell and Lagarias 2010). Of importance to plants for regulation of their cellular functions are also intermediates of chlorophyll biosynthesis as well as the redox state of the photosynthetic electron transport (Kropat, *et al.* 1997, Beck 2005, Koussevitzky, *et al.* 2007).

One chloroplast complex that is regulated by these signaling pathways is the aforementioned PSII. As shown in figure 2 it is composed of several subunits, some of which are encoded in the chloroplast (figure 2; green-colored subunits) and others in the nucleus (figure 2; grey-colored subunits). Levels of nuclear encoded factors involved in regulating PSII by controlling synthesis and assembly of subunits are efficiently controlled by their rates of transcription, whereas the expression of participating chloroplast genes is mainly regulated at the post-transcriptional level (reviewed in Goldschmidt-Clermont 1998, Leon, *et al.* 1998, Woodson and Chory 2008). In case of photosystems an interesting model of interaction between the redox state and illumination of the chloroplast has been proposed by Pfannschmidt and co-workers. Depending on light intensity there are supposed to be three different redox responses: (1) plastoquinone is responsible for exact regulation and low-light adaption, (2) the thioredoxin system reactions at medium light, and (3) the glutathione pool regulating responses to high-light and oxidative stress (Pfannschmidt, *et al.* 2001). Glutathione itself is responsible for storage and transport of reduced sulfur in many cellular processes, e.g. antioxidative defense (Tausz, *et al.* 2004). Thioredoxins are involved in the regulation of the redox environment of the cell by catalyzing thiol-disulfide interchanges. They act on their target proteins depending on ferredoxin thioredoxin reductases, NADP thioredoxin reductases or glutaredoxines. Electrons for these reactions are either provided by light or NADPH (Gelhaye, *et al.* 2005).

Focusing on *C. reinhardtii*, the following subsections will describe in more detail how synthesis of chloroplast-encoded photosystem components is regulated at different levels of expression.

1.4.1 Transcription

Expression of genes starts with the transcription of their encoding DNA. In chloroplasts of higher plants this feat is performed by RNA polymerases of different origin. One type of these enzymes, the so called "plastid-encoded plastid RNA polymerase" (PEP), is of cyanobacterial origin as is supported by the endosymbiotic roots of the chloroplast and the fact that most subunits of this RNA polymerase are encoded in the chloroplast itself. The ancestral origin of this RNA polymerase is reinforced by its susceptibility to inhibitors of bacterial transcription and its ability to complement homologous subunits in *Escherichia coli* (Severinov, *et al.* 1996, Pfannschmidt and Link 1997, Navarro, *et al.* 2000). Specificity to promoters of PEP target genes is mediated by nucleus-encoded proteins, which are called sigma-like factors due to their homology to bacterial σ^{70} factors and their ability to recognize bacterial type -35/-10 promoters (reviewed in Liere and Börner 2006, Lerbs-Mache 2011). A proposed model in *Arabidopsis* argues that transcriptional activity of the PEP as well as its specificity to its target promoters is regulated by the redox-dependent phosphorylation of a casein kinase type II plastid transcription kinase and subsequent phosphorylation of sigma factors by this kinase (Baginsky, *et al.* 1999, Schweer, *et al.* 2010).

Sigma-like factors are represented by small gene families in higher plants. Members within a family are differentially expressed according to conditions of the cell and the environment (reviewed in Lysenko 2007). The only known example for such a factor in *Chlamydomonas* is RpoD (Carter, *et al.* 2004, Bohne, *et al.* 2006).

Other RNA polymerases suggested to be involved in chloroplast transcription are phage-type enzymes with only one polypeptide chain encoded in the nucleus, which are called "nucleus-encoded plastid RNA polymerases" (NEP). They are closely related to DNA-dependent RNA polymerases from the bacteriophages T3/T7 (reviewed in Liere and Börner 2007). A nuclear encoded RNA polymerase activity in plastids was first described in spinach chloroplasts (Lerbs-Mache 1993). Further evidence was provided by the maintained transcription of chloroplast genes in plants in which distinct PEP subunits were deleted or chloroplast ribosomes were severely depleted (Hess, *et al.* 1993, Allison, *et al.* 1996, Hajdukiewicz, *et al.* 1997, Legen, *et al.* 2002). The small family of phage-type RNA polymerases in higher plants consists of three members, two of them (RpoTmp and RpoTp) are targeted to the chloroplast (reviewed in Lysenko and Kuznetsov 2005, Liere, *et al.* 2011). Interestingly, algae like *Chlamydomonas*, *Osteococcus* and *Thalassiosira* seem to lack a nucleus-encoded plastid RNA polymerase (Armbrust, *et al.* 2004, Derelle, *et al.* 2006). Investigations in

Chlamydomonas showed that there is no transcription of plastid genes in the presence of the PEP-inhibiting antibiotic rifampicin (Eberhard, *et al.* 2002). Any attempts to find a phage-type enzyme in algal plastids failed, as well as the disruption of PEP-encoding genes (Goldschmidt-Clermont 1991, Fischer, *et al.* 1996). Therefore it is likely that all chloroplast genes of *Chlamydomonas* are transcribed by the PEP (reviewed in Smith and Purton 2002).

1.4.2 RNA processing and stability

The next step of regulation of gene expression is processing and/or stabilization of newly synthesized transcripts. This is predominantly mediated by binding of *trans*-acting factors at the 5´UTR. Binding of these factors is more important in *Chlamydomonas* for RNA levels than transcription rates. There is also no example of a proven primary transcript (Salvador, *et al.* 1993, Stern, *et al.* 2010). In spinach, processing events alone can account for up to 18 different RNA molecules from a single primary transcript in the chloroplast when excluding the possibility of additional promoters (Westhoff and Herrmann 1988).

Predominant examples of processing of 5'termini in *Chlamydomonas* are the *psbA, psbB* and *psbD* transcripts. Respective mRNAs exist in two forms: a longer precursor and a predominant shorter form which results from processing of the longer one (Bruick and Mayfield 1998, Nickelsen, *et al.* 1999, Vaistij, *et al.* 2000a). The subsequent binding of regulatory proteins then effects stability. Eventually, RNA structures are altered to make them more accessible to nucleases. Subsequently, translation efficiency can be altered by association of nucleases with ribosomes (Nickelsen, *et al.* 1994, Bruick and Mayfield 1998). Processing of 5`ends can be either done by endonucleolytic cleavage or by exonucleases that partially degrade the RNA. This exonucleolytic degradation has been reported in 5` to 3` (*petD*) as well as in 3` to 5` (*atpB*) direction (Drager, *et al.* 1999, Hicks, *et al.* 2002). The existence of several different nucleases in the chloroplast allows the endonucleolytic cleavage of unprotected sequences. Presence of specific RNA-binding proteins then circumvents changes of the secondary RNA structure which would turn an unprotected transcript into an easier substrate of exonucleases (reviewed in Stern, *et al.* 2010). Another explanation suggests that these nucleolytic events facilitate coordinated translation of the *psbA, psbB and psbD* RNA. Binding of a nuclease to the pre-mRNAs would guide them to thylakoid membranes before processing and translation take place (Rochaix 1996).

RNA processing at the 3`terminus must also include nucleolytic degradation as most chloroplast transcripts have specific 3`ends but transcription termination is very inefficient (Stern, *et al.* 2010). In spinach chloroplasts, a simple model describes the degradation of 3' ends by a polynucleotide phosphorylase that degrades RNA until being stopped by secondary structures like inverted repeats (Yehudai-Resheff, *et al.* 2001). Also observed are more complicated events including cleavages by endonucleases like CSP41 or CRR2 and also protection of RNAs by capping proteins, i.e. for *psbB*, *psbD*, *petA*, *petD* and *rbcL* messages (Yang, *et al.* 1996, Boudreau, *et al.* 2000, Vaistij, *et al.* 2000b, Hashimoto, *et al.* 2003, Murakami, *et al.* 2005, Raynaud, *et al.* 2007, Johnson, *et al.* 2010). Alternatively, capping proteins might fulfill additional roles like splicing (Williams-Carrier, *et al.* 2008). *Cis* elements include binding regions for these proteins or sequences that can even stabilize the RNA in a mutant background. An example is the addition of a poly(G)-tract to the 5'UTR of the *psbD* RNA. Transcripts were still detectable when the stabilizing Nac2 factor was missing (Drager, *et al.* 1998, Nickelsen, *et al.* 1999). Importance of this stabilizing elements and factors is given again in *psbD* gene expression. Transcript levels are not as severely reduced as suggested by their synthesis rate (Klinkert, *et al.* 2005). The number of involved factors can be even increased. Polycystronic primary transcripts exist that require endonucleases for processing but also contain internal promoters (Drapier, *et al.* 1998, Rymarquis, *et al.* 2006). Examples for higher plants exist in *Arabidopsis* including HCF107, an orthologue to Mbb1 in *Chlamydomonas*, which protects 5`ends of *psbH* messages, and HCF 109 which is important for mRNAs that contain UGA codons (Meurer, *et al.* 2002, Sane, *et al.* 2005).

Similar to the processing of polycistronic messages is the event of splicing. During this process introns are removed before a transcript is translated. Chloroplast introns can be divided into the classes I or II. The most important member of class I introns in *Chlamydomonas* is represented by introns of the *psbA* gene whose splicing regulated in a light-dependent manner (Deshpande, *et al.* 1997, Herrin and Nickelsen 2004). Group II introns in this organism, present in the *psaA* gene, are even more remarkable as both of them are *trans*-spliced with three different RNAs participating in formation of the first intron. Although several introns contain specific splicing factors, other factors must be involved in this process as auto-catalytically splicing introns can be only observed at non-physiological conditions (reviewed in Goldschmidt-Clermont 2009). Splicing of *psaA* introns in *C. reinhardtii* includes a chloroplast-encoded RNA and different nucleus-encoded proteins (Goldschmidt-Clermont, *et al.* 1991, Goldschmidt-Clermont 2009). Intriguingly, besides the aforementioned light-regulation of *psbA* intron removal, there are no phenotypical differences

compared to the wild-type when intron-less genes were introduced into the chloroplast of *Chlamydomonas* (Johanningmeier and Heiss 1993, Deshpande, *et al.* 1997). Mutation of an intron-encoded open reading frame (ORF) in a psychrophilic *Chlamydomonas* species led to the loss of splicing, although the same intron splices correctly *in vitro* under the same conditions. This indicates that a maturase assists in that process (Odom, *et al.* 2004). Most identified proteins involved in splicing of transcripts in chloroplasts act very specifically on their target while a few examples seem to fulfill a more general function (examples include Ostersetzer, *et al.* 2005, Balczun, *et al.* 2006, Glanz, *et al.* 2006, Merendino, *et al.* 2006, Williams-Carrier, *et al.* 2008, Kroeger, *et al.* 2009).

Another important step in RNA metabolism is editing of organellar transcripts from cytidine to uracil residues, which is reported for most plants with the exception of chlorophytes (Stern, *et al.* 2010). Editing sites are not species specific and these events occur more often in chloroplasts than in mitochondria (Stern, *et al.* 2010). Most editing sites are situated in reading frames with a few exceptions occurring in non-coding regions. Editing besides spontaneous deamination ensures translation of a conserved amino acid (Stern, *et al.* 2010).

A final step in processing of RNAs is degradation of transcripts which do not fulfill the organelle's actual requirements. These messages are inactivated by endonucleases and do not need to be completely degraded. This is necessary as RNAs are quite often protected from degradation by inverted repeats at their 3'termini. Exceptions without those repeats most likely follow the example of the 5'ends. In these cases presence of message-specific proteins is needed (reviewed in Stern, *et al.* 2010). A list of known stabilization and processing factors in *C. reinhardtii* is presented in table 1.

Table 1: Cloned nucleus-encoded factors involved in processing and stability of plastid transcripts of *C. reinhardtii*

Factor	Homology	Target RNA	References
Mbb1	TPR protein	*psbB*	Vaistij, *et al.* 2000b
Mca1	PPR protein	*petA*	Loiselay, *et al.* 2008
Mcd1	OPR protein	*petD*	Murakami, *et al.* 2005
Mrl1	PPR protein	*rbcL*	Johnson, *et al.* 2010
Nac2	TPR protein	*psbD*	Boudreau, *et al.* 2000
Raa1	OPR protein	*psaA*	Perron, *et al.* 2004
Raa2	pseudo uridine synthetase	*psaA*	Perron, *et al.* 1999
Raa3	pyridoxamine 5`phosphate oxidase	*psaA*	Rivier, *et al.* 2001
Raa4	---	*tscA*	Glanz, *et al.* 2011
Rat1	poly-(ADP ribose)-polymerase	*tscA*	Balczun, *et al.* 2005
Rat2	---	*tscA*	Balczun, *et al.* 2005

Repeat motifs are a reoccurring theme in proteins which are involved in RNA processing. Therefore, a brief description will be given about the repeat families present in the mentioned protein examples. The shortest known repeat of 34 amino acid residues (arranged in 3-16 tandem-repeats) is present in tetratricopeptide repeat (TPR) proteins. Members of this protein family are participating in different processes by mediation of protein–protein interactions. Besides RNA processing, TPR proteins are also involved in regulation of the cell cycle or protein folding/transport. These proteins are found in various eukaryotes and prokaryotes (reviewed in D'Andrea and Regan 2003). One more amino acid is found in the repeats of pentatricopeptide repeat (PPR) proteins arranged in up to 30 tandems. They bind RNA and are especially numerous in plants although they can be found in all eukaryotes. All known examples are targeted to organelles and play an important role in regulation of gene expression (reviewed in Schmitz-Linneweber and Small 2008). The largest mentioned motif contains 38 amino acids in the octatricopeptide repeat (OPR) proteins. OPR proteins were mainly described in *Chlamydomonas*, and known examples are involved in several steps of RNA metabolism (Eberhard, *et al.* 2011).

1.4.3 Translation

Translation is the next major control level for expression of genes in the chloroplast. It is mainly regulated through initiation of translation though elongation steps can also be modulated (Marín-Navarro, *et al.* 2007). Important control mechanisms include the redox state of components involved in photosynthetic electron transport, the abundance of ATP and a pH gradient. This differentiated regulation machinery arose during the incorporation of the "cyanobacterial" chloroplast into the eukaryotic host which can already be seen in the structure of plastid 70S ribosomes. They clearly differ in structure and antibiotic susceptibility from eukaryotic ribosomes. Even though they share most features with bacterial ribosomes, some differences and unique subunits occur when comparing them to their prokaryotic counterparts. These plastid specific ribosomal proteins and additional domains in already known ribosomal proteins could have a function in organelle-specific processes as chloroplasts have a special role in integrating light-driven photosynthesis into the whole-cell metabolism (reviewed in Marín-Navarro, *et al.* 2007). General translation factors involved in elongation or release of synthesized polypeptides have been shown to be possible target of redox and phosphorylation control in a light-dependent manner (Akkaya and Breitenberger 1992, Balmer, *et al.* 2004, Wagner, *et al.* 2006). Another difference of plastidial RNAs

compared to prokaryotic RNAs influencing the translation initiation is the higher dispensability of a ribosomal binding site, the Shine-Dalgarno (SD) sequence (Hirose and Sugiura 1996). Synthesis of the D1 protein is an example for a required SD sequence in *Chlamydomonas* (Mayfield, *et al.* 1994). Additional elements for ribosome binding in the *psbA* mRNA essential for high level expression of the D1 protein were described by Mayfield and co-workers (reviewed in Barnes and Mayfield 2003). In case of *psbD* translation removal of the SD sequence resulted only in minor changes of synthesis rates (Nickelsen, *et al.* 1999). In case of the tobacco *rps2* gene a similar sequence is even a negative regulatory element (Plader and Sugiura 2003). This implies that SD sequences are not always necessary and were replaced by sequence specific factors that alter RNA structures to allow access to the ribosomes. Several nucleus-encoded *trans*-acting factors required for translation of plastid RNAs in *C. reinhardtii* have been characterized which are summarized in table 2. Other elements important for translation include the AUG start codon, potential binding by a chloroplast homologue of the *E. coli* S1 ribosomal protein and ribosomal scanning for internal start codons (reviewed in Zerges 2000).

Table 2: Cloned nucleus-encoded factors involved in plastid translation of *C. reinhardtii*.

Factor	Homology	target RNA	References
RB38/RBP40	---	*psbD*	Barnes, *et al.* 2004; Schwarz, *et al.* 2007
RB47	poly(A)-binding protein	*psbA*	Yohn, *et al.* 1998
RB60	protein disulfide isomerase	*psbA*	Kim and Mayfield 1997
Tab2	---	*psaB*	Dauvillée, *et al.* 2003
Tba1	oxidoreductase	*psbA*	Somanchi, *et al.* 2005
Tbc2	OPR protein, CRP1 (maize)	*psbC*	Auchincloss, *et al.* 2002
Tca1	---	*petA*	Raynaud, *et al.* 2007
Tda1	OPR protein	*atpA*	Eberhard, *et al.* 2011

Also important for organellar regulation of translation is the localization of translational events within the chloroplast as involved factors have been found in the stroma and in different membranes (examples can be found in Zerges and Rochaix 1998, Ossenbühl, *et al.* 2002, Somanchi, *et al.* 2005). This enables spatial organization through localization of the transcripts beside import into the chloroplast and co/post-translational localization of the proteins. This can even differ for the same transcript when it is needed for different reasons. In *Chlamydomonas* replacement of D1 in photodamaged PSII – repair synthesis - occurs co-translationally throughout the thylakoids whereas newly synthesized D1 can be found along

with *psbA* transcripts in specialized translation zones (T-zones) around the pyrenoid (Uniacke and Zerges 2007, Uniacke and Zerges 2009).

The post-transcriptional regulation of *psbA* expression is also the most extensively studied example for translation of a plastid gene in *Chlamydomonas*. *Cis*-elements include a stem-loop structure close to the start codon (Mayfield, *et al.* 1994). *Trans*-acting factors were identified by affinity chromatography using *psbA* 5`UTR. This led to the isolation of a complex consisting of at least four proteins, designated RB38, RB47, RB55 and RB60 (Danon and Mayfield 1991). The first characterized protein was RB60, a protein disulfide isomerase, later shown to be co-localized in the ER and chloroplast thylakoids (Kim and Mayfield 1997, Trebitsh, *et al.* 2001, Levitan, *et al.* 2005). RB47, a poly(A)-binding protein revealed a direct RNA binding activity, which is necessary for D1 synthesis (Yohn, *et al.* 1998). This protein localizes to "low density membranes", which harbor several other RNA binding proteins and were suggested to be the subcellular compartment in which targeted *de novo* synthesis of photosynthetic proteins takes place (Zerges and Rochaix 1998, Uniacke and Zerges 2007, Uniacke and Zerges 2009). Also for RB38, a poly(U)-binding protein, a direct interaction with transcripts was reported *in vitro*. So far, a molecular characterization of RB55 remains elusive (Barnes, *et al.* 2004). An independent approach identified an RNA binding protein of 63 kDa, RBP63, which was shown to specifically bind to an adenosine-rich region upstream of the *psbA* start codon (Ossenbühl, *et al.* 2002). Although a high level of *psbA* transcripts is already present in the dark, increased D1 synthesis in the light could be caused by increased binding activity of those RNA binding proteins (Malnoë, *et al.* 1988, Danon and Mayfield 1991). Recent results suggest a mediation of this effect by red light and/or calmodulin (Alizadeh and Cohen 2010).

As phosphorylation of RB60 in response to the ADP/ATP ratio leads to losing the RNA binding activity which is also controlled by the redox state of vicinal thiols, a picture emerges for phosphorylation-dependent redox control of D1 synthesis (Danon and Mayfield 1994b, Danon and Mayfield 1994a). One candidate electron source for a reduction of respective disulfide bridges within the protein is the thioredoxin pool, which itself is reduced by electrons transferred from PSI by ferredoxin. Indications come from Danon and Mayfield who reported a reduced translation of the *psbA* RNA in a PSI mutant (Danon and Mayfield 1994a). In the dark, RB60 is kept in its inactive form by phosphorylation. Priming and reduction of RB60 requires photoreduction of the plastoquinone pool by PSII activity showing that in the light both photosystems are required for D1 synthesis (Trebitsh and Danon 2001, Barnes and

Mayfield 2003). RB47 on the other hand is inactivated in the dark due to its oxidized state, which prevents binding to the target RNA. Reduction and activation of RB47 in the light is supposedly performed by Tba1, another oxidoreductase involved in *psbA* translation. In the dark lack of reducing equivalents leads to oxidation and inactivation of RB47 by RB60 due to the high oxidation potential of protein disulfide isomerases (Somanchi, *et al.* 2005). This provides a tool of two counteracting proteins for balancing the synthesis rates of a central subunit of PSII. A comparable system could exist in higher plants as potentially similar factors for *psbA* translation have been detected by UV crosslinking in *Arabidopsis* (Shen, *et al.* 2001).

Another example for the control of chloroplast gene expression is the translation of the *psbD* transcript in *Chlamydomonas.* At least two proteins which are encoded in the nucleus interact with the 5`UTR of the RNA. One of them is the TPR protein Nac2 whose binding is absolutely necessary for transcript accumulation (Boudreau, *et al.* 2000). The other protein is RBP40 and binding to the transcript as well as its interaction with Nac2 are required for initiation of translation (Ossenbühl and Nickelsen 2000). *Cis* elements within the RNA include the binding regions for both proteins and nucleotides in vicinity of the start codon that influence translation rates by altering the secondary structure of the RNA (Nickelsen, *et al.* 1999, Ossenbühl and Nickelsen 2000, Klinkert, *et al.* 2006). Nac2 protects the processed transcript against nucleolytic processes. Association of RBP40 to the complex/RNA evokes conformational changes around the start codon, enabling access of ribosomes and the initiation tRNA to the transcript (Klinkert, *et al.* 2004, Klinkert, *et al.* 2006). Therefore, synthesis of the D2 reaction center proteins relies on similar mechanisms of alteration of RNA structures and translation-activating proteins as described for D1 in *C. reinhardtii*, even though much less is known about the regulation of individual factors.

Processes regulating *psbD* expression are of vital importance in *C. reinhardtii* as this protein limits the accumulation rates of total PSII. Synthesis rates of the subunits D1 and CP47 in *de novo* assembly of PSII are dependent on limiting steps of D2 synthesis, whereas repair synthesis is coordinated in a different manner (Minai, *et al.* 2006). This hierarchy of *de novo* subunit assembly which highlights the importance of the Nac2/RBP40 complex is described as “control by epistasy of synthesis (CES)”. This model assumes that the subunit stoichiometry for organellar complexes which is required for assembly into a functional state has additional regulating principles beside a highly efficient degradation of unassembled subunits. Synthesis rates are supposed to be regulated by the assembly state of more

"dominant" subunits belonging to the same complex. If these "dominant" members are not bound to their complexes then they interact (in)directly with factors that regulate translation of even or less dominant subunits at the 5´UTR of their encoding transcripts in a down-regulating manner, decreasing the number of unbound/unnecessary subunits (Choquet, *et al.* 2001). This enables the cell compartment to coordinate synthesis and assembly of subunits. The hierarchy of CES for *de novo* assembly of PSII in *Chlamydomonas* starts with D2 as the most "dominant" protein. Lack of D2 protein leads to downregulation of D1, CP47, and PsbH synthesis. Next in this cascade is D1, because missing D1 proteins downregulate translation of *psbB* and *psbH* messages while D2 synthesis remains unaltered (Minai, *et al.* 2006). Evidence for this CES process has been found for all the major chloroplast complexes (Choquet and Wollman 2002). The simplest example in *Chlamydomonas* or higher plants is a reduction of *rbcL* translation rates when the small subunit of Rubisco (RBCS) is missing (Wostrikoff and Stern 2007). Inhibition at the translation level was shown by lack of transcript association to polysomes (Khrebtukova and Spreitzer 1996, Rodermel, *et al.* 1996). CES also holds true for the cytochrome b_6f-complex: a lack of subunit IV (PetD) leads to a reduction in translation of *petA* messages as described for *C. reinhardtii* and *Z. mays* (Barkan, *et al.* 1994, Kuras and Wollman 1994, Choquet, *et al.* 1998). Other examples include the ATP synthase or influence of different PSI subunits on translation of other PSI components (Girard-Bascou, *et al.* 1987, Drapier, *et al.* 1992, Stampacchia, *et al.* 1997). Intriguingly, it was shown in yeast mitochondria that CES could be also involved in assembly of several complexes (Calder and McEwen 1991, Payne, *et al.* 1991, Nakai, *et al.* 1995, Rak and Tzagoloff 2009).

While there are examples for CES in higher plants and algae this principle seems to be absent in *Synechocystis* as translation rates do not depend on the presence of other gene products and the assembly state of functional complexes. This shows the differences that arose between chloroplasts and their cyanobacterial ancestors during evolution (Yu and Vermaas 1990, Wollman, *et al.* 1999).

2. Aims of this work

Most steps of organellar gene expression are regulated at post-transcriptional levels. Beside several *cis*-elements there is a major influence of nucleus encoded *trans*-acting factors on the fate of chloroplast RNA. For an improved understanding of events in the chloroplast it is of great interest to characterize the function of these factors, as these are essential for cell metabolism and might also be of importance to applied research, e.g. the interplay between photosynthetic electron flow and hydrogen production.

As D2 is one of the major proteins of the photosynthetic apparatus, several chapters of this thesis focus on components which influence the synthesis of that protein in *Chlamydomonas*. Some characteristics of its translational activator RBP40 had been described before, e.g. interaction with the maturation factor Nac2 or binding to an uracil-rich-sequence of the *psbD* mRNA. Biochemical methods were employed that enabled the identification of RBP40. In combining that knowledge with a genetic approach, complex composition and function of its subunits were analyzed (3.1). To enrich the native RBP40 ribonucleoprotein complex an approach was developed, that is possibly applicable to all RNA-containing high molecular weight (HMW) complexes (3.2). Additionally, the regulation of association of RBP40 to HMW complexes (HMWC) was investigated, as this association is likely to be important for alterations of *cis*-elements within the target mRNA that enable D2 synthesis (3.3).

Other important components of eukaryotic photosystems are proteins that compose the light harvesting complexes. Previous experiments identified a repressing factor for translation of the encoding *lhcb* mRNAs of PSII. To achieve further insights in the regulation of this process the influence of the redox level of that factor was investigated (3.4).

Important for the function of the photosystems are also factors that play a role in repair synthesis of respective components by the stromal HSP70B chaperone complex. Therefore, specificity-mediating factors of this complex were analyzed with regard to their biochemical properties as well as their interaction with RNA-containing complexes (3.5).

The initial step of light-independent reactions of photosynthesis is catalyzed by Rubisco. This paramount complex of chloroplast metabolism and its subunits belong to the most abundant proteins on the planet. Therefore, a comparative analysis concerning the role of a factor involved in processing of the RNA encoding the large subunit of Rubisco in *Chlamydomonas* and the vascular plant *Arabidopsis* was performed (3.6).

3. Results

Included in the following section are six studies (3.1 – 3.6) that have been published in or submitted to international peer-reviewed journals. Besides a brief description of results and conclusions, the contribution of the authors to individual publications is explained.

3.1 Synthesis of the D2 protein of photosystem II in *Chlamydomonas* is controlled by a high molecular mass complex containing the RNA stabilization factor Nac2 and the translational activator RBP40. (2007)

Schwarz, C., Elles, I., Kortmann, J., Piotrowski, M. and Nickelsen, J. *Plant Cell*, 19, 3627 – 3639

In this study, the Nac2/RBP40-complex necessary for maturation and translation of *psbD* mRNA was investigated in more detail. To identify the gene of the so far only biochemically described RBP40, the protein was isolated from stromal extracts through several steps of ion-exchange and affinity chromatography and analyzed by mass spectrometry. Surprisingly, the identified gene revealed that RBP40 is identical to RB38, which was described to be part of a complex that regulates D1 synthesis through its binding activity to *psbA* mRNA (Barnes*, et al.* 2004). Interaction between RBP40 and its *psbD* target was confirmed by severely reduced synthesis levels of D2 as well as reduced association of target RNA to polysomes in RBP40 RNAi lines. Earlier work implicated that Nac2 and RBP40 are components of the same complex, which could now be confirmed by immunodetection of these proteins co-migrating in sedimentation analyses. Supporting results were achieved by CN-PAGE analyses or co-immunoprecipitation experiments. Therefore, processes of 5'UTR-mediated RNA stabilization and translation initiation are tightly coupled in *Chlamydomonas*.

My contributions to this publication were the isolation of RBP40 as well as the interaction analysis of the RBP40 protein with ribosomal and the target RNA. Furthermore, I analyzed the interaction between Nac2 and RBP40 in a mutant lacking stable *psbD* mRNA by co-immunoprecipitation. Association of the Nac2/RBP40 complex with polysomes was analyzed in sucrose density gradients. The behavior of the complex was further investigated by using an RNAi system to reduce the amount of RBP40. Ingolf Elles generated / characterized the RNAi lines, and analyzed the distribution of the Nac2/RBP40 complex in glycerol gradients. Jens Kortmann performed the CN-PAGE analysis and the protein co-immunoprecipitations in the wild-type, whereas Markus Piotrowski identified RBP40 by doing the MS sequencing of the purified stromal protein. The manuscript was written by Jörg Nickelsen, Ingolf Elles and me with final revision by Jörg Nickelsen.

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Synthesis of the D2 Protein of Photosystem II in *Chlamydomonas* Is Controlled by a High Molecular Mass Complex Containing the RNA Stabilization Factor Nac2 and the Translational Activator RBP40

Christian Schwarz,[a,1] Ingolf Elles,[a,1] Jens Kortmann,[a] Markus Piotrowski,[b] and Jörg Nickelsen[a,2]

[a] Molekulare Pflanzenwissenschaften, Ludwig-Maximilians Universität München, 80638 Munich, Germany
[b] Pflanzenphysiologie, Ruhr-Universität Bochum, Universitätsstrasse 150, D-44780 Bochum, Germany

Gene expression in chloroplasts is regulated mainly at the posttranscriptional level. In the green alga *Chlamydomonas reinhardtii*, synthesis of the D2 protein (PsbD), which is the rate-determining subunit for the assembly of photosystem II, depends on the RNA stability factor Nac2. In addition, the RNA binding protein RBP40 has been implicated in translational control via a U-rich element in the 5′ untranslated region (5′UTR) of the *psbD* mRNA. Here, we report the identification of the *RBP40* gene based on mass spectrometric analysis of its purified product. Unexpectedly, this was found to be identical to the previously described RNA binding protein RB38, which had been suggested to be involved in the regulation of D1 protein synthesis. However, we show that RBP40 binds to the *psbD* 5′UTR in a Nac2-dependent fashion both in vitro and in vivo. Molecular characterization of *RBP40* RNA interference lines confirmed that RBP40 specifically affects the initiation of D2 synthesis. Native polyacrylamide gel electrophoresis, coimmunoprecipitation, and sedimentation analyses revealed that Nac2 and RBP40 form parts of a complex of 550 kD that is displaced from the *psbD* mRNA prior to polysome assembly. Together, these data indicate that the processes of 5′UTR-mediated RNA stabilization and translation initiation are tightly coupled in *Chlamydomonas*.

INTRODUCTION

Chloroplasts originated from a cyanobacterium that formed an endosymbiotic relationship with a heterotrophically growing eukaryote. The gradual transformation of the endosymbiont into the chloroplast involved extensive gene transfer from the developing organelle to the nuclear genome of the host. Nevertheless, the modern chloroplast genome has retained a set of ~100 protein-coding genes and encodes the basic machinery for their expression, which is essentially of prokaryotic origin. Since the chloroplast-encoded gene products form multisubunit complexes with imported nucleus-encoded proteins, the requirement for tight coordination of gene expression in these two cellular compartments has resulted in the development of an intracellular communication system. This comprises nucleus-encoded regulatory factors that regulate almost all stages of chloroplast gene expression, including transcription, RNA metabolism, and splicing, as well as translation and protein complex assembly.

Translational regulation plays a central role in determining the levels of the various chloroplast proteins (Bruick and Mayfield, 1999; Zerges, 2000). The application of in vitro and in vivo approaches has allowed several *cis*-acting determinants for translation initiation to be mapped in chloroplast RNAs (Hirose and Sugiura, 1996; Bruick and Mayfield, 1999; Higgs et al., 1999; Nickelsen et al., 1999; Yukawa et al., 2007), and these probably represent the target sites for translational regulatory factors (Zerges, 2000; Manuell et al., 2004).

To date, genetic analyses have identified only a few nuclear genes whose products play a role in protein synthesis in the chloroplast. The *CRP1* gene is required for translation of *petA*/*petD* mRNA in maize (*Zea mays*) (Barkan et al., 1994; Schmitz-Linneweber et al., 2005), and *HCF107* and *HCF173* from *Arabidopsis thaliana* participate in *psbB* and *psbA* mRNA translation, respectively (Sane et al., 2005; Schult et al., 2007). Recently, a fourth factor from vascular plants, named ATAB2, was characterized in detail. ATAB2 is a novel, blue light–induced, RNA binding protein that activates the synthesis of plastid-encoded subunits of both photosystems I and II (PSI and PSII) (Barneche et al., 2006). In the green alga *Chlamydomonas reinhardtii*, its ortholog Tab2 specifically recognizes the 5′ untranslated region (5′UTR) of the *psaB* mRNA and, as a consequence, controls PsaB synthesis (Dauvillee et al., 2003). Furthermore, the algal proteins Tbc2 and Tba1 are required for the translation of *psbC* and *psbA* mRNAs, respectively (Auchincloss et al., 2002; Somanchi et al., 2005).

In vitro interaction assays using plastid RNA probes have also contributed to the identification of proteins capable of specifically recognizing distinct RNA elements within the 5′UTR of chloroplast mRNAs, and these represent good candidates for translational control factors (Bruick and Mayfield, 1999; Nickelsen, 2003). The genes for a few of these biochemically identified RNA

[1] These authors contributed equally to this work.
[2] Address correspondence to joerg.nickelsen@lrz.uni-muenchen.de.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Jörg Nickelsen (joerg.nickelsen@lrz.uni-muenchen.de).
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binding proteins have been cloned, but no mutants are currently available that substantiate their functional assignments.

One of the best-characterized examples is represented by a multisubunit complex from *Chlamydomonas* that binds to the *psbA* 5′UTR in a redox-regulated fashion (Danon and Mayfield, 1991, 1994). This complex is composed of four major polypeptides: the 63-kD protein disulfide isomerase cPDI (Kim and Mayfield, 1997), the 47-kD poly(A) binding protein cPAB1 (Yohn et al., 1998a), the novel 38-kD RNA binding protein RB38 (Barnes et al., 2004), and a 55-kD protein (RB55). RNA binding activity of the complex correlates directly with rates of D1 synthesis, and in several nuclear mutants that exhibit reduced accumulation/activity of some of these subunits, the translation of *psbA* mRNA is perturbed (Yohn et al., 1998b).

The expression of the related *psbD* gene encoding the D2 subunit of the PSII reaction center is particularly interesting, because D2 represents the starting point for the assembly of PSII as a whole (de Vitry et al., 1989; Komenda et al., 2004; Minai et al., 2006). According to the CES (for control by epistasis of synthesis) model for the temporal sequence of PSII assembly, the amount of D2 available directly determines the levels of the other component subunits of PSII via feedback control mechanisms (Minai et al., 2006).

In *Chlamydomonas*, Nac2, a 140-kD tetratricopeptide repeat protein, is strictly required for the stabilization of the *psbD* mRNA via its 5′UTR (Kuchka et al., 1989; Nickelsen et al., 1994; Boudreau et al., 2000). Furthermore, a second factor, named RBP40, binds to a U-rich translational element located 15 nucleotides upstream of the AUG start codon in vitro (Nickelsen et al., 1999; Ossenbühl and Nickelsen, 2000). Recently, the analysis of *cis*-acting suppressor mutations suggested that RBP40 functions by inducing conformational changes within the RNA region encompassing the AUG start codon and thereby regulates the early steps in translation initiation on the *psbD* message (Klinkert et al., 2006).

Here, we report the identification and characterization of the *RBP40* gene. Our genetic and biochemical data strongly suggest that the *psbD* mRNA is the primary target for RBP40 function. Furthermore, RBP40 is shown to be part of a chloroplast multisubunit complex that also contains the RNA stability factor Nac2.

RESULTS

Identification of the *RBP40* Gene

RBP40 is a soluble protein found in the stroma of chloroplasts from *Chlamydomonas*, where it forms part of a high molecular mass complex and recognizes a U-rich sequence in the 5′UTR of the *psbD* mRNA (Ossenbühl and Nickelsen, 2000). In order to isolate RBP40, a stromal fraction prepared from wild-type

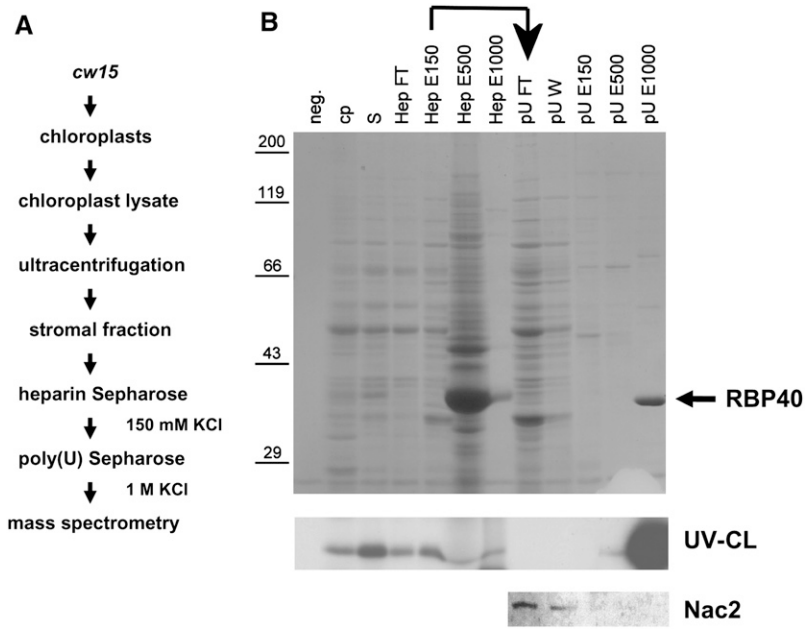

Figure 1. Isolation of RBP40.

(A) Flow chart listing the steps used to purify RBP40.

(B) SDS-PAGE and Coomassie blue staining of proteins at various stages of purification (top panel), UV cross-linking of RBP40 to radiolabeled *psbD* 5′UTR RNA (middle panel), and immunodetection of Nac2 in selected fractions by protein gel blot analysis (bottom panel). neg., negative control for RNA binding (no protein loaded); cp, chloroplast lysate; S, stromal protein fraction; HepFT, flow-through fraction from heparin-Sepharose column; HepE150, -500, and -1000, eluates obtained with 150, 500, and 1000 mM KCl, respectively; pUFT/W, flow-through/wash fraction from poly(U)-Sepharose column; pUE150, -500, and -1000, eluates obtained with 150, 500, and 1000 mM KCl, respectively. The RBP40 that eluted with high salt from poly(U)-Sepharose is marked by the arrow.

chloroplasts (Figure 1A) was loaded onto a heparin-Sepharose column, and bound proteins were eluted stepwise with KCl. Each fraction was tested for the ability to bind a radiolabeled RNA probe derived from the *psbD* 5′UTR (Figure 1B). Most of the binding activity was found in the fraction eluted with 150 mM KCl (Figure 1B, HepE150). This material was then subjected to affinity chromatography on a poly(U)-Sepharose column, with stepwise elution, and a major RNA binding activity, in the size range of ~40 kD, was eluted with 1 M KCl, indicating tight binding to the homopolymeric RNA matrix (Figure 1B, pUE1000). Gel electrophoresis and Coomassie blue staining revealed that this fraction contained one major protein of 40 kD as well as smaller amounts of material at 60 and 80 kD (Figure 1B, pUE1000).

To test whether this 40-kD protein represents RBP40, binding assays with the *psbD* probe were performed in the presence of increasing concentrations of homologous or heterologous unlabeled RNA probes as competitors. As shown in Figure 2, the 40-kD activity bound the *psbD* probe. However, in contrast with what one would expect for RBP40 (Ossenbühl and Nickelsen, 2000), it also recognized both a mutant *psbD* probe lacking the U-rich RBP40 target sequence and a *psbA* 5′UTR probe with almost equal affinity. In vivo, RBP40 activity is known to require the RNA stability factor Nac2 (Ossenbühl and Nickelsen, 2000). Therefore, we tested for the presence of Nac2 in the pUE1000 fraction using antibodies (Figure 1B, pUE1000), but none was detected. Instead, Nac2 was observed in the flow-through material containing 50 mM KCl, which itself has no RBP40 activity (Figure 1B, pUFT/W). Thus, in contrast with heparin-Sepharose, poly(U)-Sepharose chromatography does not allow copurification of the two factors.

Addition of the poly(U) flow-through to the pUE1000 fraction indeed restored high-affinity binding of RBP40 to the *psbD* 5′UTR (Figure 2). To verify that Nac2 represents the affinity-conferring component of the pUFT/W fraction, we also analyzed stromal proteins obtained from the nuclear mutant *nac2*, which lacks the Nac2 factor (Boudreau et al., 2000). When the poly(U)-Sepharose flow-through material obtained from this preparation was added to purified RBP40, the affinity of RBP40 for the *psbD* 5′UTR was reduced significantly, confirming that Nac2 is essential for high-affinity binding (Figure 2). Nevertheless, in contrast with purified RBP40 alone, the addition of *nac2* mutant material still resulted in a slight increase in the affinity of RBP40 for the *psbD* probe relative to the other two RNAs. This might indicate the presence of additional unknown factors that facilitate recognition of the *psbD* 5′UTR by RBP40. However, the data confirm that the 40-kD protein in the pUE1000 fraction represents RBP40, which is separated from the Nac2 factor during poly(U)-Sepharose chromatography.

To identify the *RBP40* gene, we subjected the 40-kD protein band to proteolytic digestion and analyzed the resulting peptides by mass spectrometry (Table 1). Surprisingly, the peptide analysis indicated that the 40-kD protein from the 1 M KCl poly(U) eluate fraction is identical to the previously described chloroplast RNA binding protein RB38, which has been implicated in regulating the translation of *psbA* mRNA (Barnes et al., 2004). That highly purified RBP40 recognizes RNA molecules in an unspecific manner (Figure 2) is consistent with the report that recombinant RB38 expressed in *Escherichia coli* binds to the 5′UTRs of

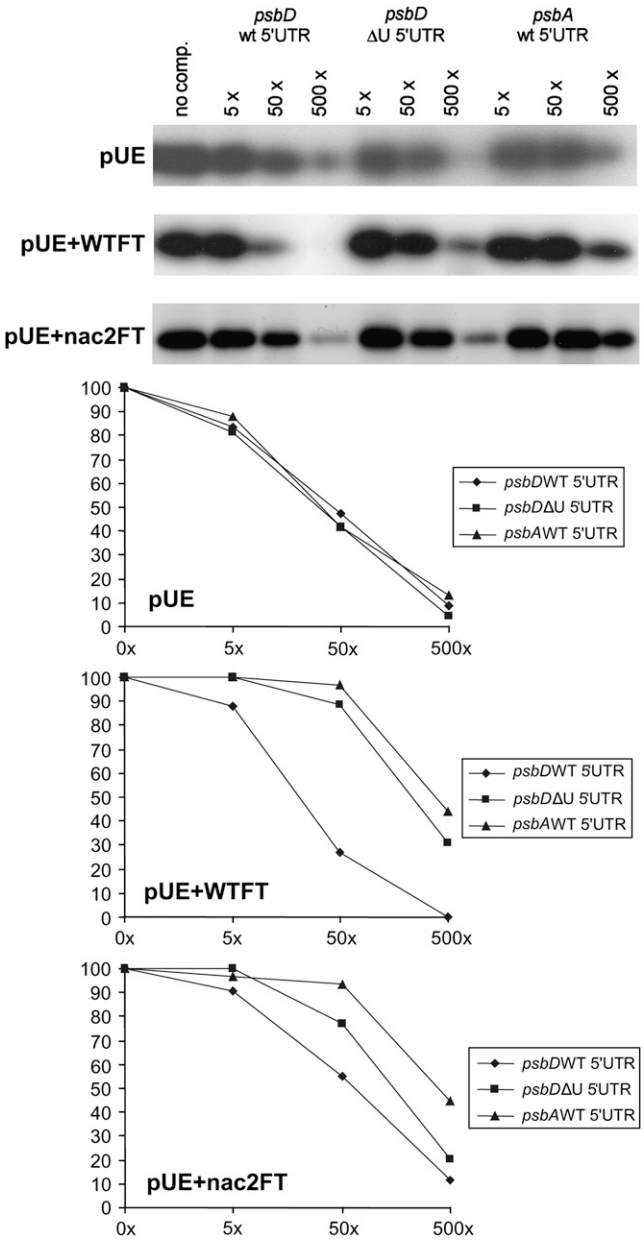

Figure 2. Nac2 Confers RNA Binding Specificity on RBP40.

(A) The pUE1000 fraction containing the purified RBP40 (see Figure 1A) alone, in combination with the wild-type flow-through fraction from the poly(U)-Sepharose column (pUE+WTFT) containing Nac2, or with the same fraction from the mutant *nac2* (pUE+nac2FT) was incubated with radiolabeled *psbD* 5′UTR RNA in the presence of a 5-, 50-, or 500-fold excess of the indicated competitor RNA and analyzed by UV cross-linking.

(B) In the graphs, the intensities of the RBP40 signals are plotted against the relative levels of the indicated competitor RNAs in the reactions, based on densitometric scanning of the autoradiograms shown in **(A)**.

Table 1. Mass Spectrometric Identification of RB40

Peptide Mass (D)	Predicted Sequence[a]	Database Hit (Swissprot Accession No.)
999	AFALWLDGR	Q6EMK7
1060	NSALWLDSR	Q6EMK7
1199	SAAPSTPELEAK	Q6EMK7
1294	SNPDEWYDNR	Q6EMK7
1328	QAAEAANWEALR	Q6EMK7

[a] Note that Leu (L) and Ile (I) cannot be distinguished.

several RNAs (Barnes et al., 2004). Since neither of these sources of RBP40/RB38 contains Nac2, these results are compatible with our finding that RBP40 only recognizes the *psbD* 5′UTR with high affinity in the presence of Nac2.

The *psbD* mRNA Is the Target of RBP40 in the Chloroplast

To identify target mRNAs for RBP40, immunoprecipitates obtained using an αRBP40 antiserum were probed with radiolabeled DNA probes derived from the 5′UTR regions of the *psbD*, *psbA*, *rbcL*, and *atpB* genes (Figure 3). All of these probes detected the corresponding transcripts in the supernatant after immunoprecipitation. However, only the *psbD* mRNA was found in substantial amounts in the precipitate, indicating that native RBP40 indeed forms a complex with the *psbD* mRNA, but not—or only to a very limited extent—with any of the other transcripts tested, including *psbA*. The preimmune serum used as a further negative control was unable to precipitate *psbD* mRNA, as expected (Figure 3).

Analysis of *RBP40* RNA Interference Lines

To confirm that RBP40 is specifically involved in regulating the synthesis of D2, *RBP40* RNA interference (RNAi) lines were generated according to Rohr et al. (2004). As described in Methods, an inverted repeat structure comprising the RBP40 coding region was cloned into the vector NE537 (Rohr et al., 2004), and 2000 transformants harboring this construct were selected on paromomycin. Of these, 63 were resistant to 10 μM 5-fluoroindole, indicating efficient silencing of the vector-encoded *Maa7* gene (Rohr et al., 2004). Subsequent chlorophyll fluorescence measurements on the 63 lines (data not shown) then identified three lines, named *40-1*, *40-5*, and *40-9*, that exhibited the most pronounced effects on photosynthetic activity. In lines *40-5* and *40-1*, the steady state levels of RBP40 protein were equivalent to 20 and 10%, respectively, of that accumulating in the wild type (Figure 4A). The greatest reduction was observed in line *40-9*, which contained <10% of the wild-type level (Figure 4A). Concomitantly, levels of D2 were reduced to 80, 30, and <5% in lines *40-5*, *40-1*, and *40-9*, respectively, indicating a strong effect of *RBP40* knockdown on PSII. Other protein complexes in the chloroplast were not affected by the RNAi construct, as judged by parallel monitoring of the steady state levels of the PsaF and AtpB subunits of PSI and ATP synthase, respectively (Figure 4A).

Interestingly, the reductions in D2 levels were not associated with any significant change in concentrations of *psbD* mRNA, indicating that RBP40 affects the synthesis or stability of PSII subunits (Figure 4B). To distinguish between these two possibilities, pulse labeling of chloroplast proteins was performed after inhibition of cytoplasmic translation with cycloheximide. D2 synthesis rates were found to be drastically reduced in RNAi lines (Figure 4C), and the degree of reduction correlated with the levels of D2 accumulation revealed by protein gel blot analysis (Figure 4A). Like the *nac2* mutant, RNAi lines also showed a reduction in labeling of the D1 protein, albeit less pronounced than in the case of D2 (Figure 4C) (Kuchka et al., 1988). Effects related to light-dependent PSII repair synthesis were minimized during these experiments, because cells from RNAi lines generally were kept in very low light. However, the virtual absence of D2 synthesis excludes the possibility that the primary effect of RBP40 deficiency is a defect in translation of the *psbA* mRNA, since, based on previous reports, D1 deficiency does not compromise the synthesis of D2 (Kuchka et al., 1988; de Vitry et al., 1989; Minai et al., 2006).

To further confirm a role for RBP40 in translation of the *psbD* message, polysomal loading of *atpB*, *psbA*, and *psbD* mRNAs was assayed (Figure 5). In the wild type, all three mRNAs migrated into the lower regions of a sucrose gradient, indicating that they were associated with polysomes and were thus being actively translated. Overall, the polysomal association of all three mRNAs in the wild type was lower than reported previously (Minai et al., 2006). However, this is likely due to the very low light conditions used in these experiments. Disassembly of polysomes by treatment with EDTA resulted in the accumulation of all three transcripts, mainly in fractions 1 to 3 at the top of the gradient; these fractions contain ribosomal subunits and smaller RNP particles (Figure 5, WT+EDTA). When the RNAi line *40-9* was analyzed, *psbD* mRNA was detected only in fractions 1 to 4, even after extended exposure of autoradiograms; none was found at polysome positions within the gradient. This finding indicates that the *psbD* mRNA is not or almost not translated in

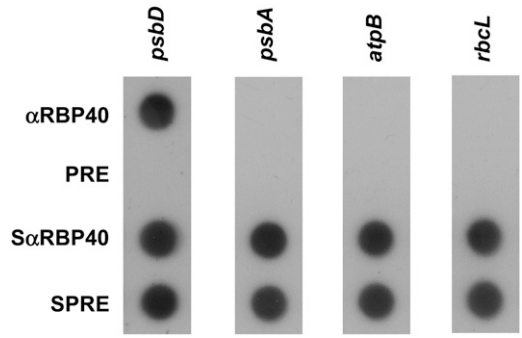

Figure 3. Coimmunoprecipitation of RBP40 and *psbD* mRNA.

Chloroplast stromal proteins were used for immunoprecipitation reactions with an αRBP40 antiserum or the preimmune serum (PRE). RNAs were extracted from precipitates (αRBP40 and PRE) and supernatants (SαRBP40 and SPRE), and equal proportions were subjected to dot-blot hybridization using the radiolabeled DNA probes indicated at top.

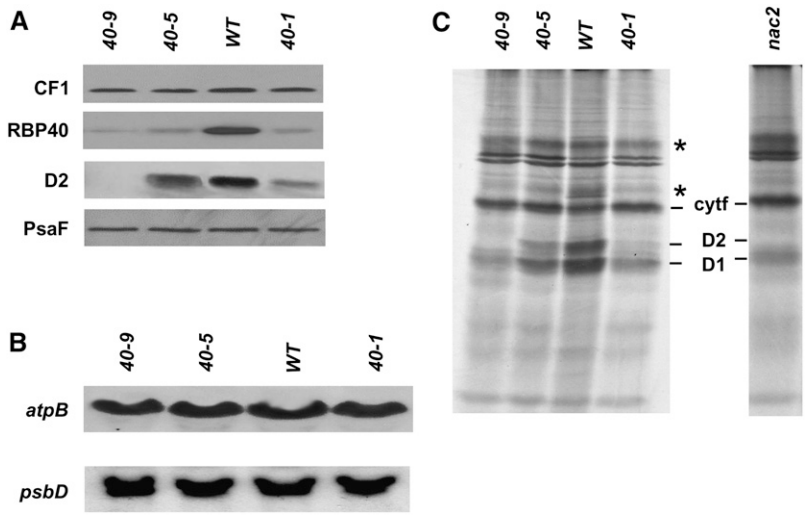

Figure 4. Molecular Characterization of *RBP40* RNAi Lines.

(A) Protein gel blot analysis of total proteins (10 μg) isolated from the indicated *RBP40* RNAi lines and the wild type was performed using antibodies raised against the proteins indicated at left.

(B) RNA gel blot analysis of *psbD* mRNA accumulation in *RBP40* RNAi lines.

(C) Total proteins from the indicated strains were pulse-labeled for 20 min with [^{35}S]sulfate and subsequently fractionated by SDS-PAGE. The positions of cytochrome *f*, D2, and D1 proteins are indicated (Klinkert et al., 2006). Fluctuations of signal intensities marked with asterisks were not seen reproducibly.

40-9. By contrast, *atpB* mRNAs migrated into the *40-9* gradient, revealing that they are actively translated in this RNAi line. The loading of *psbA* mRNA with polysomes was affected by the silencing of the *RBP40* gene in line *40-9*, although to a weaker extent compared with that of the *psbD* mRNA (Figure 5). Some mRNA was detected in the high molecular mass fraction corresponding to polysomes, but especially in fractions 4 to 6 containing monosomes, a significant decrease in *psbA* signal intensity was detectable compared with the wild type (Figure 5). This corresponds to the pulse-labeling data presented in Figure 4C, which also indicate a less-pronounced effect on D1 synthesis in the RNAi line *40-9* compared with that of D2.

Together, these data strongly support the idea that RBP40 is a *psbD*-related translation factor that acts together with the RNA stabilization factor Nac2 to regulate *psbD* gene expression at the posttranscriptional level. Furthermore, the accumulation of wild-type levels of *psbD* mRNA in the *RBP40* RNAi lines indicates that, although translational control via RBP40 requires Nac2-mediated RNA stabilization, RBP40 deficiency does not affect RNA stabilization by Nac2.

RBP40 Is Associated with Monosomes but Not with Polysomes

The Nac2 factor has been shown previously not to be associated with the polysomal fraction from *Chlamydomonas* chloroplasts, suggesting that it functions only during the early posttranscriptional stages of *psbD* expression (Boudreau et al., 2000). The same holds for RBP40, which was only observed in top fractions 1 to 8 of the polysome gradient (Figure 5). Moreover, treatment of samples with EDTA had no significant effect on RBP40 migration, suggesting that it sediments independently of polysomes (Figure 5, WT+EDTA). Nevertheless, the data did not exclude the possibility that RBP40 might interact with monosomes, for instance during translation initiation. To address this question, immunoprecipitates obtained following reaction with αRBP40 antiserum, similar to those shown in Figure 3, were hybridized with probes specific for either 16S or 23S rRNA (Figure 6). Indeed, both rRNAs were precipitated by the αRBP40 antiserum, suggesting that both ribosomal subunits, and thus monosomes, are assembled on the *psbD* mRNA while RBP40 is bound to its 5′UTR (Figure 6). However, as soon as polysomal assembly has started, RBP40 appears to leave the *psbD* message.

RBP40 and Nac2 Form Part of the Same High Molecular Mass Complex

Based on earlier sedimentation analyses, it had been speculated that Nac2 and RBP40 might form parts of the same high molecular mass complex (Boudreau et al., 2000; Ossenbühl and Nickelsen, 2000). This idea can now be substantiated by data from three independent assays. First, high molecular mass material at 550 kD is detectable using αRBP40 and αNac2 antisera after colorless native PAGE of wild-type stromal proteins (Figure 7). Furthermore, the functional interdependence of the two factors is correlated with the ability to form the 550-kD complex, as the complex could not be detected by the αRBP40 antibody in the *nac2* mutant (Figure 7).

Second, coimmunoprecipitation experiments clearly demonstrated that both factors are part of the same complex. When

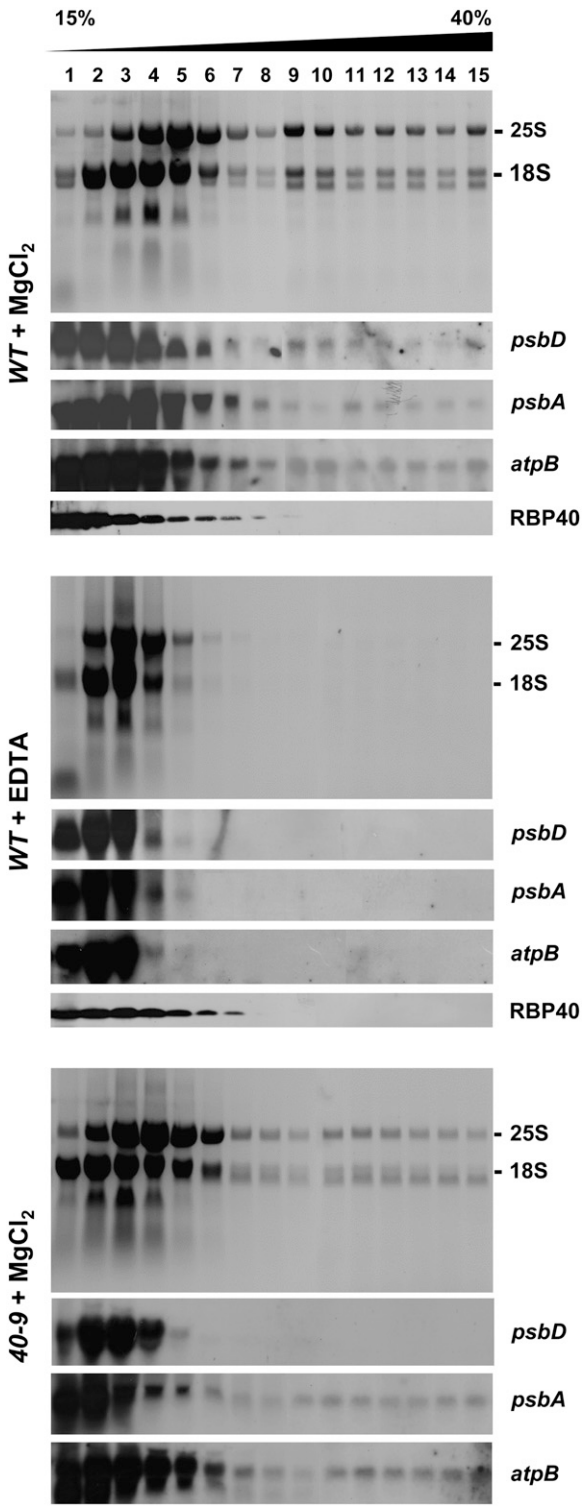

Figure 5. Polysomal Loading of *psbD* mRNA.

Whole-cell extracts from the wild type (WT+$MgCl_2$) and the RNAi line *40-9* (*40-9*+$MgCl_2$) were fractionated on 15 to 40% sucrose gradients by ultracentrifugation. As a negative control, polysomes were destabilized by the addition of EDTA (WT+EDTA). At the top of each of the three panels, the ethidium bromide–stained rRNA patterns before blotting of the gels are shown. Below, hybridization signals are shown that were obtained with the radiolabeled probes indicated at right. The sedimentation behavior of RBP40 in the wild type was followed by protein gel blot analysis of proteins from the same gradient fractions.

RBP40 was immunoprecipitated from the stromal protein extract by the αRBP40 antiserum, the Nac2 protein was detected in the precipitate (Figure 8A). Conversely, RBP40 is present in the immunoprecipitate formed upon incubation with an αNac2 antiserum (Figure 8B). As a further control, immunoprecipitations were performed using stromal proteins from the *nac2* mutant. In that case, no RBP40 protein was precipitated with αNac2 antiserum, ruling out the possibility that RBP40 might bind to the protein G–Sepharose via unrelated proteins (Figure 8B). To test whether the association of Nac2 and RBP40 depends on the presence of *psbD* mRNA, stromal proteins from a chloroplast *cis*-acting mutant called *PRB2A* were immunoprecipitated. In *PRB2A*, the PRB2 element of the *psbD* 5′UTR is mutated and, as a consequence, *psbD* transcripts do not accumulate because they are destabilized (Nickelsen et al., 1999) (see Figure 10). As shown in Figure 8B, the αNac2 antiserum was capable of precipitating RBP40 from *PRB2A*, indicating that the formation of a Nac2/RBP40 complex does not require any *psbD* mRNA.

Finally, the existence of a 550-kD complex containing both Nac2 and RBP40 was verified by demonstrating the cosedimentation of both factors after centrifugation of wild-type stromal proteins in glycerol gradients (Figure 9). When the *nac2* mutant was analyzed in the same way, RBP40 was found only in the low molecular mass fraction at the top of the gradient, again indicating that Nac2 is strictly required for the formation of the 550-kD complex and perhaps even for the assembly of subcomplexes (Figure 9; see also Figure 7). In the RNAi line *40-9*, which contains only minute amounts of RBP40 (cf. Figure 4A), a high molecular mass complex that appears slightly smaller (Figure 9, fractions 10 to 13) than the wild-type form (Figure 9, fractions 11 to 14) was observed with the αNac2 antibody. Under standard conditions, no RBP40 was detectable anywhere on the gradient. However, extended exposure of immunoblots led to the detection of tiny amounts of RBP40 in fractions 11 to 14 in line *40-9* (Figure 9, RBP40ee). This strongly suggests that loss of the 40-kD RBP40 subunit, in contrast with Nac2, does not completely destabilize the complex; instead, a smaller, RBP40-less complex of ~500 kD is observed, which contains active Nac2 and is capable of stabilizing the *psbD* mRNA (Figure 9; see also Figure 4B). However, the residual RBP40 protein still present in line *40-9* appears to associate with the 500-kD Nac2 complex, resulting in very low amounts of the normal 550 kD complex.

Earlier gel filtration analyses had revealed that *psbD* RNA–containing material was detectable throughout the 200- to 2000-kD range (Boudreau et al., 2000). This is compatible with the idea of a 550-kD complex containing Nac2, RBP40, and *psbD* RNA sequences. Treatment of the complex with RNase resulted in a reduction in its size to ~450 kD (Boudreau et al., 2000). The same shift following incubation with RNase A was observed in glycerol gradients by monitoring the distribution of both Nac2 and RBP40

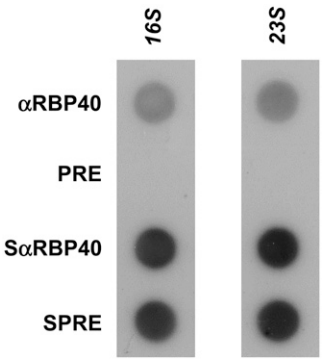

Figure 6. Association of RBP40 with Ribosomes.

Dot-blot hybridization with radiolabeled 16S and 23S rDNA probes was performed on immunoprecipitates similar to those shown in Figure 3.

using the appropriate antibodies (Figure 9). This suggests that the 550-kD complex represents a ribonucleoprotein complex. To test whether the associated RNA is derived from the *psbD* gene, we analyzed the above-mentioned mutant *PRB2A*, which does not accumulate any *psbD* transcripts. Sedimentation analysis of stromal proteins from *PRB2A* showed that the Nac2-containing complex is of the same size (450 kD) as that seen after treatment with RNase. This strongly suggests that the size reduction to 450 kD is due to the removal of *psbD* mRNA regions that remain associated with the complex during sample preparation (Figure 9). That the entire *psbD* mRNA forms an integral part of the 550-kD complex appears unlikely, because its loss would be expected to cause a larger change in molecular mass than the observed 100 kD. Most likely, endogenous RNase activities cleave away most of the complexed RNA during sample preparation and/or gradient centrifugation. However, it cannot be totally excluded that, apart from the *psbD* mRNA, other RNA molecules might be present in the Nac2/RBP40 complex, since RNase treatment also resulted in complexes that are slightly smaller than those observed in *PRB2A* (cf. fractions 6 in Figure 9).

DISCUSSION

RBP40 Specifically Recognizes the 5′UTR of the *psbD* mRNA

Here, we report the purification of the RNA binding protein RBP40 from *Chlamydomonas*, which was found to be identical to the previously described RNA binding protein RB38 (Barnes et al., 2004). Notable structural features of RBP40/RB38 include a putative chloroplast transit sequence and four repeats of a motif comprising 70 amino acids with a high percentage of basic residues. Despite limited primary sequence homology between them, these repeats appear to fold into a tertiary structure that resembles well-known RNA binding domains such as the RNA recognition motif domain (Barnes et al., 2004). As implied by these features, RB38 was shown to be imported into chloroplasts, and the recombinant protein expressed in *E. coli* exhibited RNA binding activity, showing a selective affinity only for U-rich regions (Barnes et al., 2004). Like recombinant RB38, highly purified RBP40 showed low-specificity RNA binding in vitro. However, when the RNA stability factor Nac2 was present, RBP40 bound preferentially to the 5′UTR of *psbD* RNA, suggesting that Nac2 recruits the protein into a *psbD*-specific complex or modifies the RNA binding surface of RBP40 directly (Figure 2).

Further lines of evidence indicate that RBP40 indeed represents a *trans*-acting factor required for *psbD* gene expression. First, RBP40 was shown to form a structural and functional unit with the *psbD*-specific RNA stabilization factor Nac2. By contrast, RB38 has been postulated to form part of a complex of four subunits, namely RB60, RB55, RB47, and RB38, which specifically recognizes the *psbA* 5′UTR and mediates the redox control

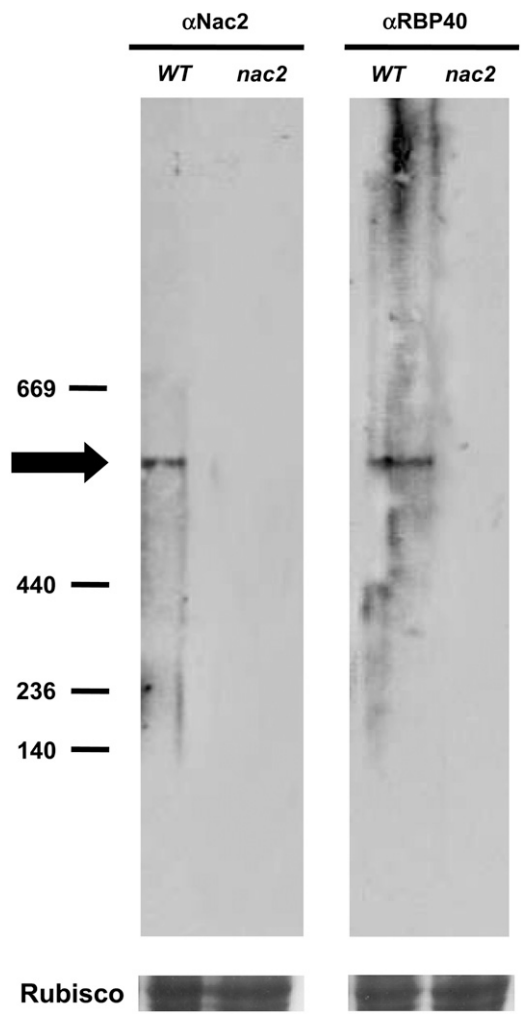

Figure 7. Native PAGE of RBP40 and Nac2.

Stromal protein fractions from wild-type and *nac2* chloroplasts were subjected to native PAGE on 8% gels and transferred to nitrocellulose filters, which were immunolabeled with either αNac2 or αRBP40 antibodies. The arrow indicates the 550-kD Nac2/RBP40 complex. Equal loading was confirmed by Ponceau red staining of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). The sizes of marker proteins are given at left.

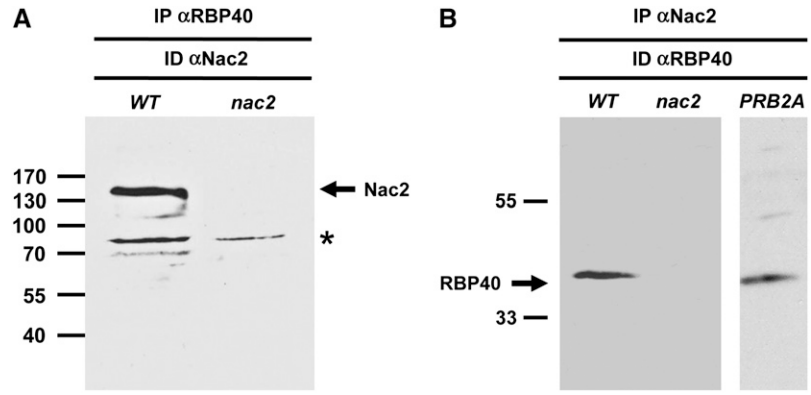

Figure 8. Coimmunoprecipitation of RBP40 and Nac2.

Stromal protein fractions from wild-type and *nac2* chloroplasts were incubated with either αRBP40 antibody coupled to protein A–Sepharose (IP αRBP40) **(A)** or αNac2 antibody coupled to protein G–Sepharose (IP αNac2) **(B)**. After elution from the matrix, the material was subjected to SDS-PAGE and immunolabeled using the same antibodies (ID αNac2 or ID αRBP40). The asterisk marks material that cross-reacts with the αNac2 antiserum.

of D1 protein synthesis (Barnes et al., 2004). This conclusion was based solely on the coelution of the four proteins from an RNA affinity column bearing *psbA* 5′UTR sequences following a single application of 0.55 M KOAc (Danon and Mayfield, 1991, 1994). An interaction between RB60 and RB47 has been demonstrated: both polypeptides were isolated from an RNase T1-resistant *psbA* 5′UTR RNA/protein complex that had been cut out of a native mobility-shift gel (Danon and Mayfield, 1991). However, neither RB38 nor RB55 was detected in this *psbA*-specific complex, arguing against the formation of a complex containing all four proteins. In this study, we show that RBP40/RB38 cosediments with Nac2 in an ~550-kD complex. Previously, we showed that the Nac2 complex is distinct from a significantly smaller complex of ~440 kD containing the RB60 protein (Boudreau et al., 2000). This indicates that RBP40/RB38 and the *psbA*-specific factor RB60 do not form parts of the same complex; therefore, it seems questionable whether RB38 is indeed involved in RB60/RB47-mediated *psbA* gene expression.

Second, *psbD*, but little or no *psbA*, *atpB*, or *rbcL*, mRNA was immunoprecipitable in substantial amounts with αRBP40 antiserum. Although we have not precisely quantitated the RNA amounts detected in these immunoprecipitates (Figure 3), the

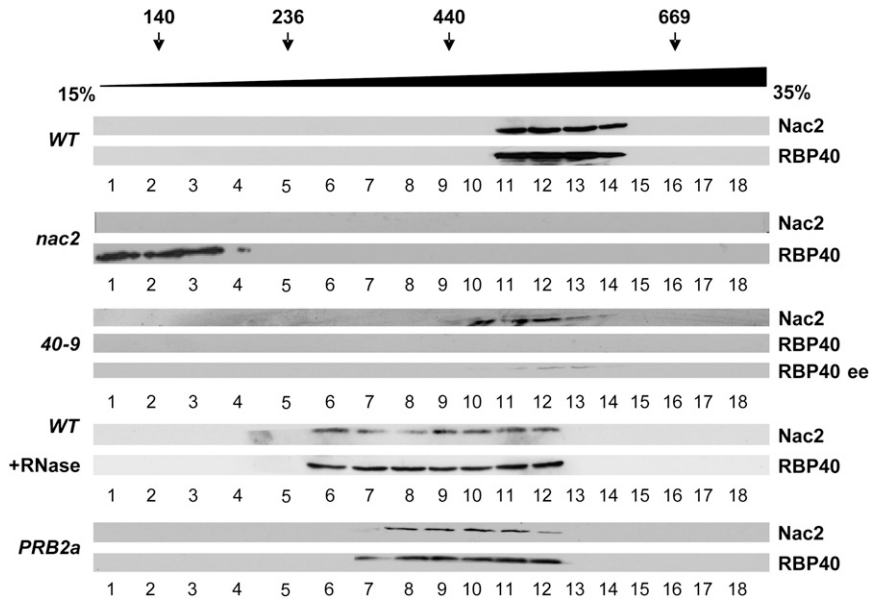

Figure 9. Formation of an RBP40/Nac2 Complex in the Absence of *psbD* mRNA.

Stromal chloroplast proteins from the strains indicated at left were centrifuged through 15 to 35% glycerol gradients. The distribution of the Nac2 and RBP40 proteins after centrifugation (at right) was monitored by protein gel blot analysis of the fractions marked below each filter strip. RBP40ee represents an extended exposure (>100-fold) of the blot shown directly above it.

dramatically obvious differences in precipitation efficiencies indicate a clear preference of RBP40 for complex formation with the *psbD* mRNA.

Finally, molecular characterization of *RBP40* RNAi lines revealed severe defects in D2 synthesis. Nevertheless, minor effects on D1 synthesis were also observed. This suggests that RBP40 might—besides *psbD*—also be involved in *psbA* gene expression. Alternatively, and more likely, reduced D1 synthesis in the *RBP40* RNAi lines is caused by a secondary effect reflecting the mode of regulation of PSII assembly/synthesis known as the CES principle (Minai et al., 2006). For instance, a strong effect of D2 deficiency on D1 synthesis has been reported for the *nac2* mutant (Kuchka et al., 1989; Nickelsen et al., 1999) (Figure 4C) and chloroplast *psbD* deletion mutants (Minai et al., 2006). This reduction is attributed to a feedback control mechanism that depends on nonassembled D1 protein accumulating in the absence of its assembly partner D2. Nonassembled D1 is sensed by an unknown mechanism, and as a consequence, D1 synthesis is reduced by processes mediated via the *psbA* 5′UTR (Minai et al., 2006). A similar situation is expected to occur in the *RBP40* RNAi lines, with partial (*40-4* and *40-1*) or almost complete (*40-9*) loss of D2 resulting in partial feedback inhibition of *psbA* translation. Indeed, an effect of RBP40 deficiency on D1 synthesis during protein pulse labeling and polysomal loading was observed (Figures 4C and 5). This is in agreement with previous reports revealing only a limited, although still significant, effect of D2 deficiency on polysomal loading of the *psbA* mRNA in a chloroplast *psbD* deletion mutant of *Chlamydomonas* (Minai et al., 2006).

Thus, together, our data support a scenario in which RBP40 is specifically or at least preferentially required for the posttranscriptional regulation of *psbD* gene expression. Whether the Nac2/RBP40 complex or parts of it are also involved in *psbA* gene expression—for instance, during feedback inhibition of *psbA* gene expression during CES regulation—remains to be clarified.

Aspects of Nac2/RBP40 Complex Formation

Three different approaches—native PAGE, coimmunoprecipitation, and sedimentation analysis—were utilized successfully to document that Nac2 and RBP40 form parts of a single complex of 550 kD. However, whether the two factors interact directly or via adapter components remains an open question. Yeast two-hybrid analysis, in vitro glutathione *S*-transferase pull down, and chemical cross-linking experiments have provided no evidence for such a direct physical interaction (data not shown). This suggests that formation of the Nac2/RBP40-containing complex requires one or more additional components that can serve as a molecular bridge between the two proteins. The sedimentation data (Figure 9) demonstrate that the *psbD* mRNA itself does not supply this function, because complex formation still occurs in the *psbD* mRNA–deficient mutant *PRB2A*. Two other lines of evidence suggest that additional polypeptides are present in the 550-kD complex. (1) Previous studies have revealed the existence of three unlinked nuclear loci that, when mutated, can suppress a defect in the RNA stability element PRB2 (Nickelsen, 2000). It was speculated that these loci encode subunits of a Nac2 complex that recognize the PRB2 element, which is located immediately upstream of the U-rich RBP40 target region in the *psbD* 5′UTR (Nickelsen, 2000) (Figure 10). (2) Treatment with RNase reduces the apparent size of the 550-kD complex by only ~100 kD, suggesting a stable protein complex of 450 kD (Figure 9).

One other aspect of Nac2/RBP40 complex formation concerns the different requirements for Nac2 and RBP40. While lack of Nac2 prevents the formation of stable high molecular mass

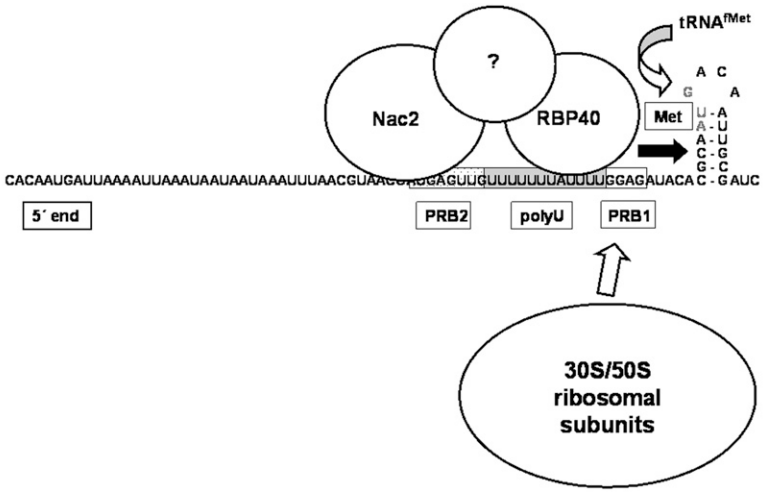

Figure 10. Working Model for the Posttranscriptional Control of *psbD* Gene Expression.

The sequence of the *psbD* 5′UTR from *Chlamydomonas* is given with the PRB2 site, the U-rich translational element boxed in gray, and the putative Shine-Dalgarno element (PRB1) (Nickelsen et al., 1999). The AUG start codon (gray letters) is marked by Met. Putative additional components of the Nac2/RBP40 complex are indicated by a question mark. The closed arrow represents the change in RNA conformation induced by RBP40, and the open arrows stand for the subsequent binding of components of the translational machinery. For further explanation, see text.

complexes containing RBP40, drastic reduction of RBP40 has only a minor effect on the Nac2-containing complex. Thus, RBP40 might be less tightly associated with the complex and/or might not represent an essential structural subunit. Whether this finding has functional implications remains to be elucidated.

Consistent with the data presented here, previous sedimentation analyses using either sucrose or glycerol gradients had revealed a Nac2 complex size of 500 to 600 kD (Boudreau et al., 2000; Ossenbühl and Nickelsen, 2000). However, additional, less-abundant Nac2 complexes of 700 to 2000 kD in size were observed using fast protein liquid chromatography gel filtration for the separation of complexes (Boudreau et al., 2000). In view of the finding that the Nac2/RBP40 complex might be associated with monosomes (Figure 6), these supercomplexes might still contain ribosomes or ribosomal subunits, thereby explaining their huge size. However, these associations are probably destabilized during the relatively long-lasting sedimentation analysis compared with the fast protein liquid chromatography procedure.

RBP40 Is Involved in Controlling the Translation of *psbD* mRNA

RBP40 recognizes a U-rich region located 15 nucleotides upstream of the *psbD* AUG start codon (Ossenbühl and Nickelsen, 2000) (Figure 10). Deletion of this U-rich stretch leads to the complete loss of D2 synthesis, while mRNA accumulation is compromised to only a minor extent. Both findings suggest that this element is required for translational control (Nickelsen et al., 1999). Moreover, detailed analyses of *cis*-acting second-site suppressor mutants have revealed a functional relationship between the U-rich stretch and an RNA stem-loop structure containing the AUG codon within the stem region. It was hypothesized that RBP40 binding alters the conformation of this region, thereby giving the ribosomal subunits access to the initiation codon (Klinkert et al., 2006) (Figure 10).

The analysis of *RBP40* RNAi lines supports the idea that RBP40 is a translational activator. In particular, the pulse labeling and polysomal loading analyses underline its significance for translation initiation (Figures 4C and 5). Interestingly, *psbD* mRNA accumulation was not affected in RBP40-deficient cell lines, indicating that Nac2 is still fully functional and, hence, can operate independently of RBP40. This is consistent with the accumulation of high molecular mass Nac2 complexes in the absence of RBP40. By contrast, absence of Nac2 leads to the loss of translational activity, even when the *psbD* mRNA is artificially stabilized by the insertion of poly(G) stretches into the *psbD* 5′UTR (Nickelsen et al., 1999), indicating that Nac2 is required for the RBP40 function. Thus, the Nac2/RBP40 complex fulfills two distinct functions. First, a subcomplex containing Nac2 but lacking RBP40 is sufficient to stabilize the *psbD* mRNA, probably via the PRB2 element (Figure 10). Then, the complete Nac2/RBP40 holocomplex of 550 kD mediates the subsequent steps of *psbD* gene expression (i.e., translation initiation via an interaction with the U-rich element) (Klinkert et al., 2006). Future work will focus on the identification of the predicted additional subunits of the complex by genetic and biochemical means, with a view to obtaining a more complete picture of the regulatory network controlling *psbD* gene expression in *Chlamydomonas*.

Strikingly, a recent characterization of the spatial organization of PSII synthesis and assembly processes has revealed that RBP40 is localized to a specialized chloroplast subcompartment near the pyrenoid, called the T zones (Uniacke and Zerges, 2007). This further underlines the role that RBP40 plays for the de novo biosynthesis of PSII.

METHODS

Culture Conditions

Chlamydomonas reinhardtii strains were grown to a density of 2×10^6 cells/mL in Tris-acetate-phosphate (TAP) medium (Harris, 1989) containing 1% sorbitol. RNAi lines were grown in low light (<5 mE·m^{-2}·s^{-1}). Chlorophyll content was determined following acetone extraction as described before (Klinkert et al., 2006).

Purification of RBP40 and Peptide Sequencing by Mass Spectrometry

Chloroplasts from cell wall–deficient strains carrying the *cw15* mutation were isolated from a discontinuous Percoll gradient (45 to 75%) as described previously (Zerges and Rochaix, 1998). To prepare chloroplast stromal fractions, isolated chloroplasts were osmotically lysed in hypotonic buffer (10 mM Tricine/KOH, pH 7.8, 10 mM EDTA, and 5 mM 2-mercaptoethanol) by repeated pipetting. Insoluble material was removed by ultracentrifugation for 30 min at 100,000*g* through a 1 M sucrose cushion in hypotonic buffer in an SW40 rotor (Beckman).

An aliquot of the supernatant containing 10 to 15 mg of stromal protein was then applied to a 5-mL heparin–Sepharose 4B (GE Healthcare) column equilibrated with buffer I (50 mM KCl, 10 mM Tricine/KOH, pH 7.8, and 10 mM EDTA). Bound proteins were eluted using a discontinuous salt gradient (150 mM, 550 mM, and 1 M KCl in buffer I). Proteins eluting at 150 mM KCl were desalted using Amicon Ultra centrifugal filtration devices (Millipore) with a 10-kD molecular mass cutoff according to the manufacturer's instructions.

The protein solution (in buffer I) was then applied to a 2-mL poly(U)–Sepharose 4B (GE Healthcare) column equilibrated with buffer I. The column was washed with 3 volumes of buffer I, and bound proteins were eluted with a discontinuous salt gradient (150 mM, 550 mM and 1 M KCl in buffer I).

The different fractions were tested for the presence of RNA binding activity by assessing their ability to bind to 5′UTR sequences from *psbD* RNA in UV cross-linking assays. Prior to use in UV cross-linking assays (see below), all protein fractions were dialyzed against RNA binding buffer (30 mM Tris-HCl, pH 7.0, 50 mM KCl, 5 mM $MgCl_2$, and 5 mM 2-mercaptoethanol). Protein concentrations were determined using the Bradford assay (Bio-Rad).

For mass spectrometric peptide sequencing, RBP40-containing gel pieces were treated with trypsin (sequencing grade; Promega) and the resulting peptides were analyzed on a Q-TOF2 mass spectrometer (Micromass) as described (Piotrowski and Volmer, 2006).

Production of Antiserum against RBP40

An *RBP40* cDNA was isolated after screening of a cDNA library prepared from wild-type cells (Boudreau et al., 2000). A DNA fragment encoding the first 191 amino acids of RBP40 was amplified from this cDNA by PCR with the primers 5′-GGATCCGCCGCGGCGCACCCCCCTGG-3′ (RBP40-BamHI-5′) and 5′-GTCGACGCTGTCCAGCCACAGCG-3′ (RBP40-SalI-3′). The fragment was cloned into the expression vector pGEX4T1 (via the *Bam*HI and *Sal*I restriction sites present in the two primers). Overexpression of this construct in the *Escherichia coli* strain BL21 and purification of the resulting RBP40–glutathione *S*-transferase fusion protein were

performed according to the manufacturer's protocol using glutathione–Sepharose 4B (GE Healthcare). A polyclonal antiserum was produced by immunizing rabbits with this protein fraction (Biogenes).

Analysis of Nucleic Acids and Proteins

Pulse labeling of total cell proteins with [^{35}S]sulfate, isolation of algal nucleic acids and proteins, and RNA gel blot and protein gel blot analyses were performed essentially as described previously (Klinkert et al., 2006). Signal intensities in four independent protein gel blot analyses were densitometrically quantified using Scion Image software (http://www.scioncorp.com).

Generation of *RBP40* RNAi Lines

To create RBP40-deficient mutants of *Chlamydomonas*, we used the RNAi system described previously by Rohr et al. (2004). For the generation of an inverted repeat construct specific for RBP40 RNA, a 573-bp fragment corresponding to the 5′ coding sequence of *RBP40* was amplified with PCR using the *RBP40* cDNA as a template with the primers RBP40-BamHI-5′ and RBP40-SalI-3′, which add *Bam*HI and *Sal*I restriction sites, respectively. A longer 800-bp fragment containing an additional 227 bp of the coding sequence that functioned as a spacer for the inverted repeat was amplified using the primers RBP40 RNAi-H-E-S (5′-AAGCTTGAATTCCTGACCTTGAGACGTGC-3′) and RBP40 RNAi3′ (5′-GAGCTCGTCGACCTAGTAGCGGGCGC-3′), adding *Hin*dIII, *Eco*RI, *Sal*I, and *Sac*I restriction sites to the sequences. These two fragments were ligated and cloned as an inverted repeat (with the central spacer) into the *Eco*RI site of the vector NE537 (which is located in the inverted repeat of the *Maa7* gene for the β-subunit of Trp synthase) using the *E. coli* strain XL-1 Blue as a host (Rohr et al., 2004).

Cells of the *cw15* strain were transformed with the resulting construct, kept for 2 d in liquid culture (TAP + 1.5 mM L-Trp) in dim light, and then plated on TAP plates containing 5 μg/mL paromomycin (to select for transformants) and 1.5 mM L-Trp. At intervals of 2 weeks, colonies were transferred to TAP plates containing 5 and then 10 μM 5-fluoroindole in the dark. 5-Fluoroindole–resistant clones were screened for high chlorophyll fluorescence phenotypes and then subjected to molecular analysis.

In Vitro Synthesis of RNA and UV Cross-Linking of RNA to Proteins

DNA templates for the in vitro synthesis of *psbD* and *psbA* leader RNA probes were generated by PCR using appropriate primers: for *psbD* RNA (the wild-type and the *ΔU* mutant sequences of the *psbD* mRNA corresponding to positions −74 to +18 relative to the AUG), 5′-ACCGATCGCAATTGTCAT-3′ (3131) and 5′-TAATACGACTCACTATAGGGACACAATGATTAAAATTAAA-3′ (2126); and for *psbA* RNA (the wild-type sequence of the *psbA* mRNA corresponding to positions −91 to +13 relative to the AUG), 5′-GTAATACGACTCACTATAGGGTACCATGCTTTTAATAGAAG-3′ (T7-*psbA*5′) and 5′-GATCCATGGTCATATGTTAATTTTTTTAAAG-3′ (2054). In vitro transcription of RNA, UV cross-linking of RNA to protein, and quantification of binding signals were performed as described previously (Klinkert et al., 2006). Radiolabeled RNA and nonlabeled competitors were mixed prior to the addition of proteins to competition experiments. Quantification of competitor RNAs was performed by measuring the incorporation of low levels of radioactivity into transcripts. Signal intensities in competition experiments were densitometrically quantified using Scion Image software.

Coimmunoprecipitations of Proteins and RNA

Chlamydomonas chloroplasts were isolated as described above and resuspended in lysis buffer (10 mM Tricine/KOH, pH 7.8, 10 mM EDTA, 5 mM 2-mercaptoethanol, and Roche Complete mini protease inhibitor cocktail). Membranes were pelleted by centrifugation for 30 min at 100,000*g* through a 1 M sucrose cushion in an SW40 rotor (Beckman). The resulting supernatant constituted the stromal fraction used for subsequent immunoprecipitation experiments. To minimize nonspecific interactions, this supernatant was first incubated with 250 μL of protein A–Sepharose (GE Healthcare) in lysis buffer for 1 h at 4°C. For coimmunoprecipitations, αRBP40 IgGs cross-linked to 10 mg of protein A–Sepharose were added to the pretreated stromal fraction, and the mixture was incubated overnight at 4°C. The beads were then washed 10 times in Tris-buffered saline–BSA (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% BSA, and Roche Complete mini protease inhibitor cocktail). Bound proteins were released from the beads in 5× SDS loading buffer (50% glycerol, 125 mM Tris-HCl, pH 7.0, 5% SDS, 0.05% bromophenol blue, and 150 mM 2-mercaptoethanol) for 15 min and subsequently subjected to immunoblotting analysis. For immunoprecipitations using αNac2 IgGs, the same protocol was followed, except that protein G–Sepharose was substituted for protein A–Sepharose.

Coimmunoprecipitations of RNA were performed in the presence of 0.5 μg/μL yeast tRNA and 1 unit/μL RNasin (Promega). The RNA was isolated by extraction with phenol-chloroform after the addition of SDS to 0.5%. Equal proportions of RNA samples were transferred to nylon membranes using a dot-blot manifold (Schleicher and Schüll). To avoid membrane saturation effects, total RNA amount per dot was restricted to 2.5 μg. Subsequently, membranes were hybridized with radiolabeled DNA probes comprising the 5′UTRs of the indicated genes, which were PCR-amplified using appropriate oligonucleotides. As a loading control, all blots were finally hybridized with the *psbD* probe.

Sedimentation Analysis in Glycerol Gradients

For sedimentation analysis, isolated chloroplasts were hypotonically lysed in 20 mM Tricine/KOH, pH 7.8, 55 mM KCl, 3 mM EDTA, 5 mM ε-amino caproic acid, 5 μg/μL tRNA, 80 units of RNasin, and 0.05% BSA. Aliquots equivalent to 1.5 mg of stromal proteins were then loaded onto 15 to 35% glycerol gradients and centrifuged for 18 h at 180,000*g* in an SW40 rotor (Beckman). The gradient was fractionated into 18 0.5-mL samples, and 40 μL of each was used for immunoblotting analysis.

Polysome Purification in Sucrose Gradients

Polysomes were purified as described previously (Mussgnug et al., 2005). Cells (7.4×10^8) were recovered by centrifugation, broken in a freeze–thaw cycle, resuspended in 1 mL of polysome extraction buffer (200 mM Tris-HCl, pH 9.0, 200 mM KCl, 35 mM $MgCl_2$, 25 mM EGTA, 0.2 M sucrose, 1% Triton X-100, and 2% polyethylene-10-tridecyl-ether), supplemented with inhibitors (0.5 mg/mL heparin, 100 mM 2-mercaptoethanol, 100 mg/mL chloramphenicol, 1 mM 1,10-phenanthroline, and 0.5% [v/v] Complete mini protease inhibitor cocktail either with or without EDTA [Roche]), and immediately centrifuged at 4°C for 20 min at 10,000*g*. The supernatant was supplemented with sodium deoxycholate to a final concentration of 0.5% and layered onto a linear gradient composed of 15 to 40% sucrose in cushion buffer (40 mM Tris-HCl, pH 9.0, 20 mM KCl, 30 mM $MgCl_2$ or 1 mM EDTA, and 5 mM EGTA). The gradients were centrifuged for 225 min at 100,000*g* at 4°C. Aliquots of collected fractions were either used for immunoblot analysis or supplemented with 0.5% SDS and 20 mM EDTA before RNA isolation by phenol/chloroform extraction. RNA was dissolved in nuclease-free water and separated on a 1% agarose-formaldehyde gel.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AJ271460 (*Nac2*) and AY124882 (*RPB40*/*RB38*).

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3.2 Enrichment of native, high molecular weight ribonucleoprotein complexes from chloroplast by consecutive gel filtration steps. (2010)

Schwarz, C. and Nickelsen, J. *Endocyt. Cell Res.*, 20, 89 – 94

With the experience of having analyzed several high molecular weight ribonucleoprotein complexes, we established a general method to solve the problem of low level accumulation of soluble RNA-binding complexes in and above the Rubisco size range. To circumvent their concealment by complexes that are more abundant, we isolated stromal complexes larger than 1 MDa in size in a first gel permeation chromatography step. Addition of RNase leads to an exclusive shift of regulatory complexes containing RNA towards lower molecular weight fractions as exemplarily shown for the Nac2/RBP40 complex. After a second consecutive gel permeation step, proteins of these ribonucleoprotein (RNP) complexes can be isolated from fractions <1 MDa, which are free of non-RNP proteins.

I compared the behavior of native complexes in gel filtration analyses. The manuscript was written by Jörg Nickelsen and me with final supervision by Jörg Nickelsen.

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Technical notes

Enrichment of native, high molecular weight ribonucleoprotein complexes from chloroplasts by consecutive gel filtration steps

Christian Schwarz and Joerg Nickelsen
Department Biology I, Molecular Plant Sciences, Ludwig Maximilian University, Munich, Germany;
Correspondence to: joerg.nickelsen@lrz.uni-muenchen.de

Chloroplast gene expression is regulated at various steps including the synthesis, stabilization/ maturation and translation of plastid mRNAs. Nucleus-encoded gene products have been shown to be involved in this regulation via the formation of complexes with their cognate chloroplast RNA targets. As regulatory key factors, they usually accumulate to only low amounts. Here, we describe a strategy for the enrichment of native high molecular weight chloroplast ribonucleoprotein particles from *Chlamydomonas reinhardtii* by using consecutive gel filtration steps.

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Introduction

Recent years have seen the identification of numerous nucleus-encoded factors which participate in chloroplast RNA metabolism and translation (for review see Stern et al. 2010). Accumulating evidence indicates that most of these factors form parts of multi-subunit, high molecular weight (HMW) complexes with their target RNAs (RNPs), which often accumulate to only low amounts (Nickelsen 2003; Schwarz et al. 2007; Pfalz et al. 2009; Johnson et al. 2010). For instance, the Nac2 protein is involved in stabilization of the chloroplast *psbD* mRNA in the unicellular green alga *C. reinhardtii*, while the RNA binding protein RBP40 is required for translation of the same mRNA. We have previously shown that these two factors assemble into *psbD* mRNA containing HMW complexes (Boudreau et al. 2000; Schwarz et al. 2007). However, high-abundant plastid HMW complexes containing no RNA, for instance the ribulose-1,5-bisphosphate-carboxylase/oxygenase-complex (Rubisco), interfere with preparative biochemical analyses of native RNP complexes by using density gradient centrifugation and/or size exclusion chromatography (SEC).

We have previously drafted an experimental procedure which allows for the selective enrichment of native RNP particles (Bohne et al. 2009). The basic strategy is given in Figure 1A. Provided that at least one of the protein constituents of the RNP complex can be followed by either its activity or immunological means, a first purification step involves separation of complexes via SEC. This procedure is favorable when compared to other methods due to its relatively low processing time of ca. 1h as compared for instance to 18h during a single density gradient centrifugation step. This minimizes degradation processes on both RNA and protein moieties of RNPs.

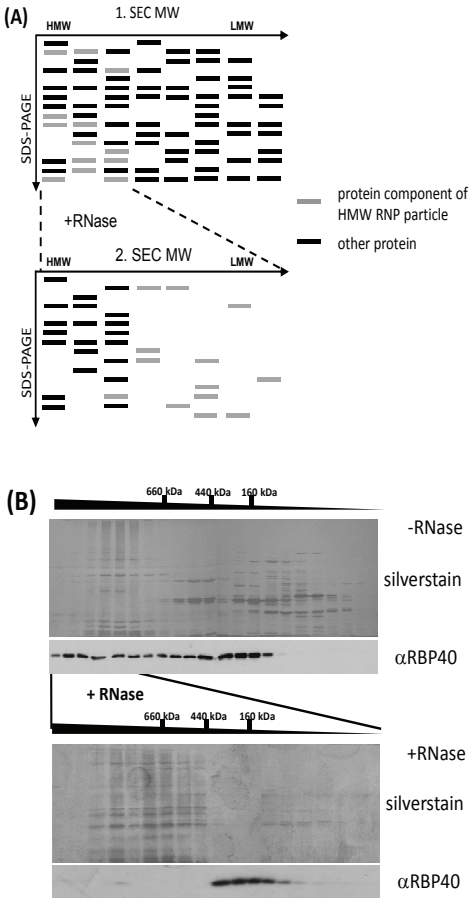

Figure 1: (A) Scheme for enrichment of RNP proteins by digesting HMW complexes with RNase A (adapted from Bohne et al. 2009). (B) SEC behavior of RBP40 before and after RNase treatment. 1 mg of stromal proteins from *C. reinhardtii* was separated on a Superose 6 10/300GL column. 1% of each fraction was analyzed in parallel by silver staining for overall protein content and Western blotting for RBP40 distribution (upper part). After determination of RBP40 distribution, only fractions eluting above ~ 1000 kDa were concentrated and treated with RNase A before a second round of SEC. Most of the remaining RBP40 now appears in lower molecular weight fractions.

Further specific enrichment of RNP particles is then achieved by treating only pooled fractions containing HMW complexes with RNase and a subsequent second SEC step. Addition of RNase leads to degradation of the nuclease-accessible parts of RNAs from HMW RNP particles and, therefore, to a drastically reduced size of the residual RNP complexes which now contain only their protein subunits and those RNA parts which are protected by bound proteins. Since RNase inhibitors reduce the efficiency of this treatment, they are omitted throughout the whole procedure. Other non RNA-containing HMW protein complexes are RNase-insensitive and, therefore, elute at the same position as in the first SEC step. Consequently, proteins that are part of RNP complexes are specifically enriched in lower molecular weight (LMW) fractions after the second SEC step due to the removal of accessible RNA parts from RNP particles (Figure 1A; Bohne et al. 2009). RNase does not interfere with downstream analyses of the RNP complexes, because it is separated from these complexes due to its smaller size during the second SEC step.

When the Nac2/RBP40 complex is exemplarily analyzed by following this strategy, a huge variety of RNP complex forms, ranging in size from 2000 – 160 kDa, is detected after elution from the first gel filtration column. This broad range reflects degradation processes acting especially on the nuclease-accessible parts of the *psbD* mRNA during the preparation of stromal extracts and/or assembly intermediates of the Nac2/RBP40 complex (Figure 1B). Rationalizing that especially the largest HMW complexes represent intact RNPs containing most of the *psbD* mRNA, only RNPs in the size range of 2000 – 1000 kDa are further processed. Thereby, most of the high-abundant interfering complexes like the Rubisco enzyme are readily excluded from the preparation. After RNase treatment and the second SEC step, the residual Nac2/RBP40 complex elutes in fractions corresponding to a size of 450 – 150 kDa which is in agreement with previous analyses using glycerol gradient centrifugation (Figure 1B; Schwarz et al. 2007). Silver staining of elution fractions reveals an approximate 10fold enrichment of the Nac2/RBP40 complex after the second SEC step when RBP40 signal intensities per protein amount are compared to the first SEC step (Figure 1B). Below, we provide the detailed experimental protocol for the enrichment of the Nac2/RBP40 complex which is likely to be applicable to the analysis of other chloroplast RNPs (for a flow chart, see Figure 2).

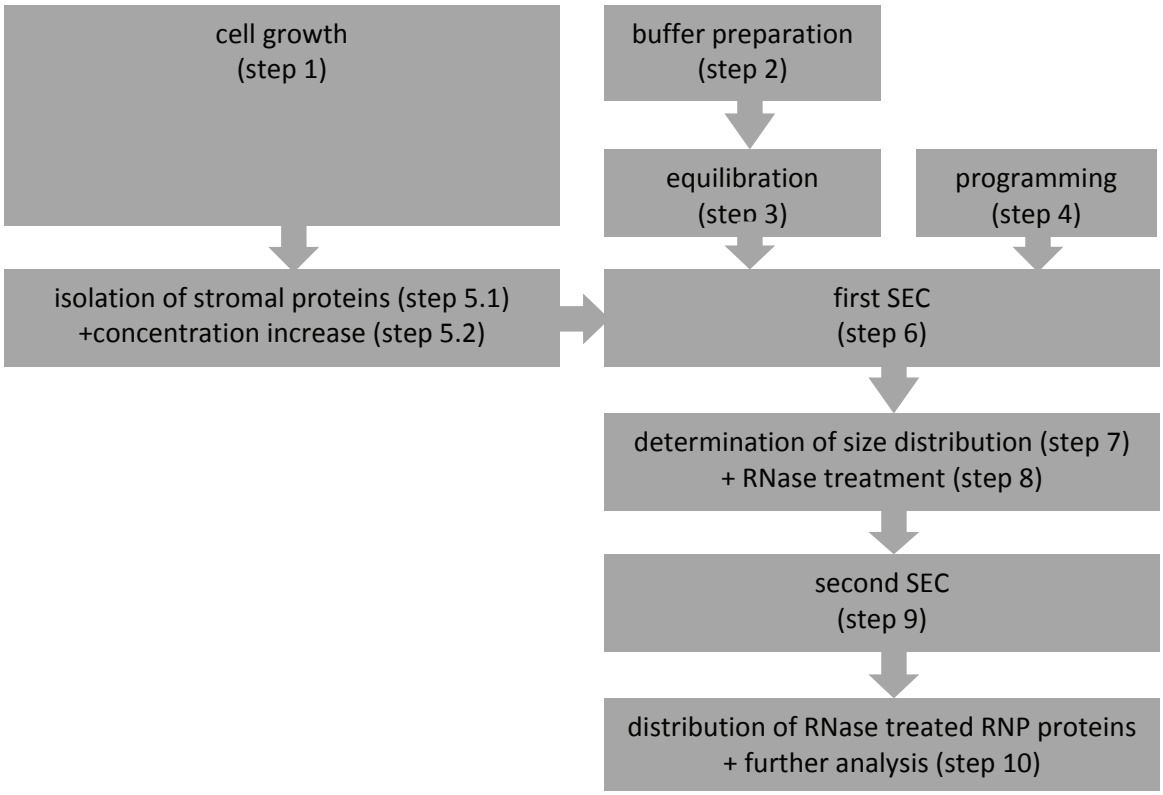

Figure 2: Flow chart for selective enrichment of high molecular weight RNP complexes.

Cultivation of cells

Media and growth conditions for cells are given by Harris (2009; Step 1). For isolation of chloroplasts, cell-wall reduced strains are used. Alternatively, autolysin treatment can remove the cell-wall. Conditions for cell treatment with this enzyme have to be established for every strain individually (Harris 2009).

Preparation of columns and chromatography system

Column material is chosen based its optimal resolution in the size range of HMW RNPs, i. e. 2000 – 100 kDa. These

criteria are met, e.g., by matrices like Superose or TSK-Gel (GE Healthcare and Tosoh Bioscience, respectively). The chromatographic processes are under the control of the Unicorn operating software using an ÄKTApurifier 10 system (GE Healthcare).

Buffers need to be filtered to ensure that no solid particles enter the chromatography system. A convenient method for larger buffer volumes is vacuum filtration using appropriate membranes. Degassing is also necessary as air entering the system can significantly affect the experiment, e.g. larger air bubbles passing through the system make photometric measurements impossible or carbon dioxide could acidify the buffer (Step 2). After washing with water, the column is equilibrated with the buffer that was prepared in the second step (Step 3). The Unicorn software is used in parallel for programming the SEC steps in accordance with column parameters (Step 4).

Isolation of stromal proteins

Isolation of chloroplasts and stromal proteins is done as described by Zerges and Rochaix (1998). Since RNA-protein interactions are intrinsically unstable, buffers and equipment must be cooled at 4°C throughout the entire experiment. It is also important to work as fast as possible to limit degradation processes (Step 5). Proteins are concentrated by ultrafiltration using Amicon Ultra filters (Millipore, Step 5.2).

Size exclusion of untreated and treated stromal proteins

Using the program from step 4 and proteins from step 5, soluble complexes are separated according to their hydrodynamic volume (Step 6). Only the HMW fractions of the complexes, supposed to contain intact RNA, are collected (Figure 1; Step 7). As SEC leads to dilution of the initial sample, the increased sample volume has to be reduced for the next gel filtration step by ultrafiltration. Concomitantly, the HMW RNPs are subjected to RNase treatment (Step 8).

The resulting mixture is then applied to a second round of SEC using parameters from step 6 (Step 9). Having lost their nuclease-accessible RNA parts, residual RNPs now have a different size exclusion behavior. In contrast, non-RNA containing HMW complexes will not change size and, thus, are specifically separated from RNP complexes.

Further analysis of proteins

Fractions collected in step 9 are used for downstream applications and/or further purification steps, like mass spectroscopic analyses or immunoprecipitations, after determination of the size distribution of residual RNPs (lower part of Figure 1B; Step 10; see also Watkins et al. 2007; Kroeger et al. 2009). By using two coupled gel filtration steps, RNA interacting proteins can be investigated in a rapid fashion since fast separation procedures at low temperatures minimize the risk of degradation caused by RNases / proteases or disassembly of less stable complexes.

Acknowledgement

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Box

Step 1 - Cell growth – Cells from the cell wall-reduced strain CC406 are grown in liquid TAPS medium to a density of less than $2 \cdot 10^6$ cells/ml to harvest them in the log phase (Harris 2009). Cultures are grown at 23 °C with shaking at 120 rpm and a light intensity of 30 µE m^{-2} s^{-1}.

Step 2 - Buffer preparation – SEC buffer used in the analysis of the Nac2/RBP40 complex contains 20 mM tricine/KOH, pH 7.5, 50 mM KCl, 5 mM ε-aminocaproic acid and 2.5 mM EDTA. When estimating the necessary amount of buffer, consider for each step:

equilibration	5 column volumes
elution	1 column volume
washing	2 column volumes .

All experimental procedures from now on are performed at 4°C. Adjustment of pH value and removal of solid particles by vacuum filtration are carried out at the same temperature because pH value and gas solubility are both dependent on temperature (Weast 1975). The same holds for any other buffer.

Step 3 - System/column equilibration – Use the parameters for a Superose 6 10/300 GL column, with resolution limits of 5 – 5000 kDa for globular proteins, in the Unicorn software's method editor:

Alarm Pressure limit	1.5 MPa
Column volume	23.6 ml
flowrate	0.5 ml / min
Injection Valve	Inject
EndMethod	5 column volumes

Complete buffer exchange can be assumed when monitored values, e.g. conductivity, stay stable for two to five column volumes. Residual proteins from previous runs, including RNases, are removed during the equilibration with 5 column volumes of SEC buffer.

Step 4 - Programming – During equilibration, SEC parameters can be added in the method editor of the Unicorn software.

Pump mode	Normal
Flow rate	0.3 – 0.5 ml/min
AutozeroUV	to normalize the baseline of the UV photometer
Injection valve	Inject (0.5 ml) Load (23.5 ml)
Fractionation	0 – 24 ml (includes void volume fractions that are discarded later)
Fraction	0.75 ml (starts a new fraction every 0.75 ml, can be changed to any desired volume, results in 30 fractions for that fractionation)
EndMethod	72 ml (continues buffer flow after fractionation until total volume of 72 ml to remove residual material)

Step 5 - Isolation of stromal proteins – Chloroplasts are isolated from ~ $2 \cdot 10^9$ CC406 cells. Cell harvest is usually done in fixed-angle rotors, while all other centrifugation steps are performed in swing-out rotors. The method of Zerges and Rochaix (1998) can be used at 4°C with the following modifications. To isolate intact chloroplasts from the strain CC406, 15 ml of resuspended cells (in isotonic solution) are lysed by incubating them with the detergent saponin for exactly five minutes (mix one volume of cells in isotonic solution with one volume of 10 % saponin in isotonic solution). The lysate is sedimented by centrifugation (4000g, 10 sec).

4x isotonic solution	1.2 M sorbitol
	20 mM magnesium chloride
	40 mM tricine pH 7.5

The resulting sediment is resuspended in 8 ml of hypotonic solution and layered on a 10 ml 45% / 10 ml 75% Percoll step gradient, which is then centrifuged (6000 g, 20 min). Intact chloroplasts migrate to the interphase between the Percoll solutions. To remove Percoll, the chloroplasts are taken from the gradient and diluted with twice the volume of isotonic solution before being centrifuged (5000 g, 10 min). After complete removal of isotonic solution chloroplasts are lysed osmotically by applying ~1 ml of hypotonic solution (add protease inhibitors, for instance Roche CompleteMini Inhibitor cocktail) and repeated pipetting using a 1000µl pipette, e.g. Gilson Pipetman P1000 and Greiner 740291 tips.

hypotonic solution	10 mM EDTA
	10 mM tricine/KOH pH7.5

Insoluble material is removed by layering ~ 1ml lysate on 12 ml cushion of 0.6 M sucrose in hypotonic solution and subsequent centrifugation (100000 g, 30 min). The resulting supernatant is considered as stromal proteins (Zerges and Rochaix 1998). Protein content is determined as described by Bradford (1976).

Step 5.2 - Concentrating stromal proteins – As hypotonic lysis usually leads to large sample volumes, lysates have to be concentrated to fulfill sample volume restrictions for SEC. The sample contains 1 mg of stromal proteins, while the sample volume has to be less than 1% of the total column volume (~250 µl for a Superose 6 10/300GL column). The lysate is then concentrated by using Amicon Ultra filters with an exclusion limit of 30 kDa (Millipore).

Step 6 - First SEC – Make sure that the system is in an idle position. Inject the stromal proteins into the sample loop by using the INV-907 mixer of the ÄKTApurifier system. The first chromatography step is then started with the parameters that were programmed at step 4.

Step 7 - Determinations of protein distribution – Aliquots of 1 % of the eluted fractions are separated on SDS-PAGE gels. Overall protein distribution is checked with silver staining of the gels as described by Heukeshoven and Dernick (1988). Distribution of RBP40 is followed by Western blotting using available antibodies (upper part of Fig. 1B).

Step 8 - RNase treatment – As seen in Figure 1B, HMW complexes containing RBP40 elute above ~1000 kDa. These fractions are pooled and their volume is adjusted to sample loop restrictions by applying ultrafiltration in the presence of 200 µg RNase A (see step 5.2). During the concentration procedure, RNase degrades the accessible RNA parts of RNPs.

Step 9 - Second SEC – Having lost most of their RNA, the RNP particles have a reduced molecular weight and hydrodynamic volume. This results in a different SEC pattern when performing the chromatography again using the RNase treated samples and the same Unicorn parameters as in step 6.

Step 10 - Determination of protein distribution from RNase treated HMW complexes – The additional elution fractions of step 9 undergo the same procedures as described in step 7. The expected change of the RBP40 elution profile is confirmed as shown in Figure 1B.

3.3 An intermolecular disulfide-based light switch for chloroplast *psbD* gene expression in *Chlamydomonas reinhardtii*. (2011)

Schwarz, C., Bohne, A.V., Cejudo, F.J. and Nickelsen, J. *Plant J.*, (submitted)

We analyzed the regulation of the interaction between Nac2 and RBP40 and its influence on D2 synthesis in *C. reinhardtii*. Light-dependent translation of the *psbD* mRNA is regulated by the interaction between a poly(U)-sequence within the 5'UTR of the transcript and RBP40. Deletion of that sequence abolishes RBP40 binding and D2 synthesis. Suppressor mutants exhibiting point mutations in the *psbD* 5'UTR, which are able to restore D2 synthesis but not binding to RBP40, showed lower increase of light-dependent D2 synthesis in comparison to the wild-type. SEC analyses showed that RBP40 dissociated from the Nac2 complex in the dark and this disassembly heavily depends on the redox state of the single cysteine residue of RBP40. These results could be confirmed by addition of a reducing agent to isolated stroma of light-grown strains before SEC as well as in 2D redox gels that show a Nac2-dependent intermolecular disulfide bridge in RBP40 in the light. Reduction of this disulfide bridge could also be achieved *in vitro* by addition of NTRC, a NADPH-dependent thioredoxin reductase with an additional thioredoxin domain. Therefore, our data indicate a light-controlled formation of the NAC2/RBP40 complex and resulting D2 synthesis levels. Additionally, these processes require cross-talk with the energy state of the cell as NTRC is involved in carbon metabolism.

I was involved in this work by performing size exclusion filtrations of all analyzed strains as well as redox dependent two-dimensional gels using antibodies raised against proteins of interest. Additionally, I performed the analysis of protein synthesis and steady state RNA levels as well as the S-alkylation of RBP40. Alexandra-Viola Bohne carried out the cloning and expression of full-length recombinant RBP40 as well as UVX experiments. Francisco Javier Cejudo provided the recombinant NTRC enzymes. The manuscript was written by all authors supervised by Jörg Nickelsen.

An intermolecular disulfide-based light switch for chloroplast *psbD* gene expression in *Chlamydomonas reinhardtii*

Christian Schwarz[1], Alexandra-Viola Bohne[1], Francisco Javier Cejudo[2], Jörg Nickelsen[1]*

[1]Molekulare Pflanzenwissenschaften, Biozentrum LMU München, Grosshaderner Str. 2-4, 82152 Planegg-Martinsried, Germany
[2]Instituto de Bioquimica Vegetal y Fotosintesis, University of Seville and Consejo Superior de Investigaciones Cientificas, Avda Americo Vespucio 49, 41902 Seville, Spain

*For correspondence (fax: +49 89 2180 9974773, email: joerg.nickelsen@lrz.uni-muenchen.de)

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The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instruction for Authors is: Jörg Nickelsen (joerg.nickelsen@lrz.uni-muenchen.de)

Abstract

Expression of the chloroplast *psbD* gene encoding the D2 protein of the photosystem II (PSII) reaction center is regulated by light. In the green alga *Chlamydomonas reinhardtii*, D2 synthesis requires a high-molecular-weight complex containing the RNA stabilization factor Nac2 and the translational activator RBP40. Based on size exclusion chromatography (SEC) analyses, we provide evidence that light control of D2 synthesis depends on the dynamic formation of the Nac2/RBP40 complex. Furthermore, 2D redox SDS-PAGE assays revealed an intermolecular disulfide bridge between Nac2 and Cys_{11} of RBP40 as the molecular basis for attachment of RBP40 to the complex in light-grown cells. This covalent link is reduced in the dark, most likely via the NADPH-dependent thioredoxin reductase C (NTRC), suggesting crosstalk between chloroplast gene expression and chloroplast carbon metabolism during dark adaption of algal cells.

Introduction

Owing to the endosymbiotic origins of the chloroplast, its gene expression machinery is basically of prokaryotic origin. During the evolutionary development of chloroplasts, however, this machinery was extensively modified by the recruitment of nucleus-encoded regulatory factors which nowadays constitute an intracellular network dedicated to the coordination of gene expression in the nucleus and the organelle (for a recent review see Barkan 2011). While recent years have seen the identification and characterization of a number of these *trans*-acting factors, much less is known about their precise molecular modes of action with regard to light-dependent regulation.

In this context, the idea of redox control of chloroplast gene expression has attracted much attention, since it provides an appealing basis for a direct link between photosynthetic activity and the expression of photosynthesis-related chloroplast genes (Dietz and Pfannschmidt 2011). Indeed, many elements of chloroplast gene expression, including RNA transcription, stabilization, processing and splicing, and translation have been shown to be affected directly or indirectly by the redox state of the organelle (for an overview see Barnes and Mayfield 2003). Translation, however, appears to represent the rate-limiting step for the synthesis of chloroplast-encoded proteins (Eberhard et al. 2002; Zerges and Hauser 2009).

In the green alga *Chlamydomonas reinhardtii*, synthesis of the large subunit of ribulose 1,5 bisphosphate carboxylase/oxygenase (Rubisco) encoded by the *rbcL* gene has been shown to be regulated via the redox state of the chloroplast glutathione pool, which in turn is modulated by light-induced oxidative stress (Irihimovitch and Shapira 2000). Interestingly, the RbcL protein possesses an intrinsic non-specific RNA binding activity located within its N-terminal region (Yosef et al. 2004). It has therefore been postulated that the binding of RbcL to its own mRNA blocks its translation if either its redox-controlled interaction with the chloroplast chaperone system or Rubisco subunit assembly is disturbed (Cohen et al. 2005).

The most elaborate – but also most controversial – model for redox-controlled translational regulation in chloroplasts has been described in *C. reinhardtii* for the *psbA* gene that encodes the D1 protein of the photosystem II (PSII) reaction center (Barnes and Mayfield 2003; Zerges and Hauser 2009). This model postulates that redox-controlled binding of a protein complex to the 5´ UTR of the *psbA* mRNA leads

to recruitment of ribosomes. The heart of this complex is the RNA-binding protein RB47, whose activity is modulated by RB60, a disulfide isomerase homolog (Kim and Mayfield 2002). RB60 was shown to form intermolecular disulfide bonds with RB47 *in vitro*, suggesting tight cooperation of these factors also *in vivo* (Alergand et al. 2006). It was proposed that light-dependent reduction of the involved thiol groups in RB60 provides the molecular basis for light-dependent increases in D1 synthesis (Trebitsh et al. 2000).

We have previously shown that the expression of the chloroplast *psbD* gene in *C. reinhardtii* is under the control of a high-molecular-weight (HMW) complex containing the RNA stabilization factor Nac2 and the translational activator RBP40 (Schwarz et al. 2007). Furthermore, translation of *psbD* mRNA depends on an U-rich element within its 5´ UTR which serves as a binding site for the translational activator RBP40 (Nickelsen et al. 1999; Ossenbühl and Nickelsen 2000). Deletion of this element (ΔU) results in the complete loss of D2 synthesis (Nickelsen et al. 1999; see also Fig. 1a, b) but is partially restored in genetically selected second-site suppressor lines, namely *suΔU+9*, *suΔU-3* which harbor point mutations in a downstream RNA stem-loop structure encompassing the AUG start codon (Klinkert et al. 2006). In these lines, the *psbD* mRNA can be translated in the absence of RBP40 binding, leading to a model in which Nac2-assisted binding of RBP40 to the U-rich element affects the RNA conformation at the initiation codon, and thereby makes the initiation site accessible to the translational machinery (Schwarz et al. 2007). Thus, both RBP40 and the RNA stem-loop have the capacity to form a molecular switch that regulates *psbD* gene expression and, as a direct consequence of the so-called CES (control by epistasis of synthesis) process, the accumulation of the entire PSII in *C. reinhardtii* (Minai et al. 2006).

Here, we report on the molecular mechanisms that underlie light-controlled regulation of *psbD* gene expression via the RBP40/RNA stem-loop switch. We provide evidence showing that RBP40 is required for this control and that the light-dependent formation of the active Nac2/RBP40 complex is mediated by the establishment of an intermolecular disulfide bridge between the two factors. The redox state of this connection appears to represent a key determinant for D2, and therefore PSII, synthesis.

Results

Light regulation of D2 synthesis depends on the RBP40/RNA stem-loop switch

To test whether the RBP40/*psbD* RNA stem-loop switch is involved in the well-known light-dependent regulation of D2 synthesis in *C. reinhardtii,* we analyzed light-dependent D2 synthesis rates in the deletion strain *ΔU* (which lacks the U-rich element) and the suppressor lines *suΔU-3* and *suΔU+9* which have lost the RBP40 binding site but contain a less stable RNA stem-loop (Malnoe et al. 1988; Klinkert et al. 2006). In pulse-labeling experiments, wild-type cells grown in the light exhibited an 2.8-fold increase in rates of D2 synthesis relative to cells which had been adapted to dark conditions for 38 h (Fig. 1a, b). As described previously, in the dark the ΔU strain showed no D2 protein synthesis, while both suppressor lines exhibited reduced D2 synthesis compared to the wild-type (Nickelsen et al. 1999). Moreover, in the light, D2 expression increased only 1.9-fold in *suΔu-3* and *suΔU+9* strains (Fig. 1a, b). These results suggest that in the suppressor lines both the overall rate of *psbD* mRNA translation and the degree of induction of D2 synthesis by light are affected. Northern analyses verified that the observed differences are due to translational effects, since no significant alterations in *psbD* mRNA levels occurred under the conditions tested (Fig. 1c). In conclusion, these findings suggest that RBP40 is required for efficient regulation of D2 synthesis by light since bypass of RBP40 function in the suppressor lines results in reduced levels of light control. This supports the hypothesis of a light switch which is constituted by the negatively acting *psbD* mRNA stem-loop at the AUG start codon and RBP40 which activates translation by changing the conformation of this RNA structure.

RBP40 contains a single Cys residue

How then does light affect this molecular switch? Redox reactions have been postulated to play critical roles during light activation of chloroplast gene expression (Barnes and Mayfield 2003; Dietz and Pfannschmidt 2011) and, interestingly, RBP40 had been identified as a target for glutathionylation under conditions of oxidative stress in a proteomic analysis in *C. reinhardtii* (Michelet et al. 2008). Inspection of the amino acid sequence of RBP40 revealed the presence of only a single cysteine residue at amino acid position 11 (Cys_{11}) which could serve as a target for

glutathione binding (Fig. 2a). However, Cys_{11} is located within the predicted N-terminal transit sequence of RBP40 which should be cleaved off upon import by the chloroplast and, thus, should not be present in the mature protein (Barnes et al. 2004). To test for the presence of a Cys residue in mature RBP40, stromal proteins from *C. reinhardtii* were treated with the thiol-alkylating reagent PEG5000-maleimide for various times, and subsequently analyzed by immunoblotting. As shown in Figure 2b, an alkylation-dependent size shift of RBP40 was observed, indicating that Cys_{11} is indeed still present in the mature RBP40. This implies either that RBP40 is imported into the chloroplast by an alternative pathway which does not involve N-terminal processing of proteins or it contains an unusually short transit sequence of less than 11 amino acid residues (Schwenkert et al. 2011). Both possibilities are compatible with previous *in vitro* import experiments, which have detected no size change in RBP40 after transport into chloroplasts (Barnes et al. 2004).

To test whether the redox state of Cys_{11} directly affects the RNA-binding activity of RBP40, UV crosslinking experiments with full-length recombinant RBP40 (rRBP40) and a *psbD* 5´ UTR RNA probe were performed under different redox conditions (Fig. 2c, supple. Fig 1). The addition of neither oxidized (GSSG) nor reduced (GSH) glutathione to the reaction mixture had any influence on RNA recognition (Fig. 2c). Moreover, alkylation with NEM had no significant effect on RNA binding, indicating that binding of RBP40 to RNA is not dependent upon the redox state of Cys_{11}.

Light- and redox-dependent formation of the Nac2/RBP40 complex

We have previously shown that RBP40 forms a complex with the RNA stabilization factor Nac2 and that this interaction specifies recognition of the *psbD* 5´ UTR by RBP40; on its own RBP40 binds to any RNA, at least *in vitro* (Ossenbühl and Nickelsen 2000; Barnes et al. 2004; Schwarz et al. 2007). We therefore wished to know whether the interaction with Nac2 is affected by the redox state of Cys_{11}. To this end, we analyzed the distribution of stromal RNA/protein (RNP) complexes by size-exclusion chromatography (SEC, Johnson et al. 2010; Schwarz and Nickelsen 2010). When wild-type cells were grown in the light, the previously described Nac2/RBP40 complex was identified by both Nac2 and RBP40 antibodies in the range of 550 kDa (Schwarz et al. 2007, Fig. 3a, fractions 6-9). In addition, even larger complexes in the range of 1000 kDa were detected only with the RBP40 antibody (Fig. 3a, fractions 4 and 5). These latter complexes have not been observed in

previous experiments using time-consuming glycerol gradient centrifugation for RNP complex separation, probably because they are relatively labile. Since RNA co-immunoprecipitation experiments have previously shown that ribosomal RNA can be precipitated by an RBP40 antibody these RBP40-specific HMW complexes might represent associations with ribosomes/ribosomal subunits during the initiation phase of translation when Nac2 has already left the *psbD* mRNA (Schwarz et al. 2007). This idea is further supported by data revealing that the ribosomal protein S1 and, thus, at least the small ribosomal subunit partially co-elutes with these larger RBP40-containing complexes (Fig. 3a).

Intriguingly, when dark-adapted cells were analyzed, RBP40 accumulated only in the low molecular weight (LMW) range, peaking at ~160 kDa (Fig. 3a, fractions 9-15). Concomitantly, the Nac2 signal shifted towards the later fractions 8-10, corresponding to a complex of smaller size in the range of 440 kDa (Fig. 3a). This suggests that, in the dark, most of RBP40 is detached from the Nac2 complex and, as a consequence, *psbD* mRNA translation would be turned down. Hence, the dynamic formation of the Nac2/RBP40 complex could provide the molecular basis for the observed light-dependent regulation of D2 synthesis (Fig. 1). To determine whether formation of this complex is redox-dependent, we performed SEC analysis on RNP complexes from light-grown cells in the presence of reduced glutathione. As shown in Figure 3a, these reducing conditions resulted in the detachment of RBP40 from the Nac2 complex, although the effect was less pronounced than that observed in dark-grown cells (Fig. 3a, fractions 8-15). Nevertheless, the data strongly suggest that the redox state does have a critical role in Nac2/RBP40 complex formation.

Moreover, SEC analysis of RNP complexes from the suppressor line *suΔU+9* revealed the presence of HMW Nac2/RBP40 complexes similar to the situation in the wild-type (Fig. 3b). This indicates that interaction of RBP40 with its cognate binding site on the *psbD* 5´ UTR is not a prerequisite for Nac2/RBP40 complex formation, which is consistent with the earlier finding that this complex can form even in a mutant strain lacking the *psbD* mRNA (Schwarz et al. 2007).

The observed redox control of RBP40 association with the Nac2 complex raises the question whether photosynthetic electron flow is directly involved in controlling the synthesis of D2. To check this, two photosynthetic mutants with defects in either PSII or PSI were examined with regard to formation of Nac2/RBP40 complexes in the light. In *mbb1*, a nuclear factor is mutated that is required for the stabilization of the

chloroplast *psbB* mRNA encoding the CP47 subunit of PSII (Vaistij et al. 2000), as Nac2 is for *psbD* stability. As shown in Figure 3b, the distribution of both Nac2 and RBP40 following SEC analysis resembled that found for light-grown wild-type cells, indicating that the absence of PSII does not affect Nac2/RBP40 complex formation per se. In the *psaA trans*-splicing mutant *raa1*, PSI is absent, causing severe oxidative stress when cells are grown in the light (Merendino et al. 2006). Under these conditions, partial disassembly of the Nac2/RBP40 complex was observed as indicated by the shift of both the Nac2 and the RBP40 signal towards lower molecular weights during SEC (Fig. 3b). This suggests that oxidative stress might lead to down-regulation of D2 and, consequently, of PSII synthesis.

Light- and redox-dependent disulfide bridge formation between Nac2 and RBP40

The data obtained so far support the idea of a light-dependent control of Nac2/RBP40 complex formation which might involve Cys_{11} of RBP40. To test this more directly, two-dimensional SDS-PAGE analyses were carried out in which stromal proteins were first fractionated by SDS-PAGE in the absence of reducing agents, i.e. preserving preformed disulfide bridges (Ströher and Dietz 2008), and then orthogonally electrophoresed under reducing conditions. Consequently, polypeptides that contain no S-S groups in their native state come to lie on a diagonal across the second-dimension gel, while intermolecular or intramolecular disulfide bridges cause deviations from the diagonal to the left or right, respectively (Ströher and Dietz 2008).

When stromal proteins from light-grown wild-type cells were analyzed by following this procedure, some RBP40 was detected on the diagonal, but substantial amounts were also found in the HMW range up to ca. 170 kDa (Fig. 4a). When stromal proteins were pretreated with reduced glutathione, no such HMW signals were detectable, indicating that RBP40 forms an intermolecular disulfide bridge via its single cysteine Cys_{11} (Fig. 4b). On the other hand, most Nac2 was found on the diagonal at 140 kDa, but lesser amounts migrated in the range of the RBP40 signal at 170 kDa (Fig. 4a). Reduction prior to electrophoresis in the first dimension eliminated this 170 kDa HMW form, confirming that its formation is redox-dependent (Fig. 4b). These findings are consistent with the existence of a direct disulfide bridge between Cys_{11} in RBP40 and one of the several Cys residues present in Nac2 (Fig.

S2). The analysis of the *nac2-26* mutant, which fails to accumulate any Nac2 protein, further substantiated this idea: no HMW RBP40 signals were obtained in this strain (Fig. 4c).

Strikingly, HMW RBP40 signals were also lacking when stromal proteins from dark-adapted wild-type cells were assayed, suggesting that the disulfide bridge linking Nac2 and RBP40 is reduced in the dark (Fig. 4b). Furthermore, the Nac2 signal appeared to be shifted towards lower molecular weight on the right side of the diagonal, suggesting enhanced formation of intramolecular disulfide bridges in Nac2 in the dark (Fig. 4b).

We also analyzed Nac2/RBP40 disulfide bond formation in the genetic backgrounds used to study complex formation by SEC. In the suppressor *su⊿U+9*, Nac2 and RBP40 signals at 170 kDa were actually enhanced, indicating efficient binding of RBP40 to Nac2 despite the absence of its cognate binding site on the *psbD* 5'UTR (Fig. 4c). Furthermore, several additional intermediate RBP40 signals appeared whose nature remains elusive. In *mbb1*, enhanced Nac2/RBP40 binding was also detected, verifying that the effect seen in the *nac2-26* mutant is not due to a deficiency in PSII but is Nac2-specific (Fig. 4c). Finally, reduction of the Nac2-RBP40 disulfide bridge was found to occur in light-grown *raa1* cells, suggesting that oxidative stress leads to down-regulation of D2 synthesis via the Nac2/RBP40 redox switch (Fig. 4c). In conclusion, these data reveal a clear correlation between Nac2/RBP40 complex formation as visualized by SEC analysis and the formation of a disulfide bridge between Nac2 and RBP40.

Blue or red light do not affect Nac2/RBP40 complex formation

In higher plants, both chloroplast transcription and translation have been shown to be regulated by exposure to low levels of blue light (Gamble and Mullet 1989; Barneche et al. 2006). In particular *psbD* gene transcription depends on a blue-light-responsive promoter element which is recognized by a specific sigma factor, namely sig5 (for an overview see Lerbs-Mache 2011). However, in the chloroplast of *C. reinhardtii* only a single sigma factor has been shown to operate, and accordingly no obvious changes in *psbD* mRNA levels were observed under the light conditions applied in this work (Carter et al. 2004; Bohne et al. 2006, Fig. 1c). Nevertheless, we tested whether exposure of dark-adapted cells to low-level blue or red light induces Nac2/RBP40-disulfide bridge formation. As shown in Figure 5, irradiation with neither red nor blue

for 3 h does induce formation of the Nac2/RBP40 complex. This suggests that disulfide bridge formation is not dependent on signal relays activated by blue or red light.

Chloroplast NTRC might be involved in reduction of the Nac2/RBP40 disulfide bridge in the dark

The emerging picture of light-dependent regulation of D2 synthesis postulates a central role for the redox state of the Nac2/RBP40 complex. In the light, assembly of this complex requires formation of a disulfide bond between the two proteins. In the dark or under oxidative stress, this bond is reduced and, consequently, RBP40 is detached from Nac2. While oxidative stress is likely to lead to the previously observed glutathionylation of RBP40 (Michelet et al. 2008), it has remained obscure how reduction in the dark can be achieved.

One candidate for this role is the recently identified NTRC (chloroplast NADPH-dependent thioredoxin reductase C) enzyme (Serrato et al. 2004). NTRC reduces disulfides in the dark using electrons from NADPH, which is generated by the oxidative pentose phosphate pathway (Kirchsteiger et al. 2009). To test the possibility that NTRC might be involved in redox regulation of the Nac2/RBP40 complex in the dark, 2D redox PAGE was performed with stromal proteins from light-grown cells that had been preincubated with recombinant rNTRC from the cyanobacterium *Anabena* sp. PCC 7120 in the presence of 250 µM NADPH (Fig. 6a). Whereas NADPH alone had no effect on the covalent link between Nac2 and RBP40, the disulfide bridge was reduced when the cyanobacterial enzyme was added. In contrast, rNTRC from rice had no effect on the Nac2/RBP40 complex (data not shown), suggesting that specific recognition of the disulfide target has diverged during evolution. Nevertheless, in *C. reinhardtii* it does appear that the Nac2/RBP40 complex represents a target for chloroplast NTRC, which may therefore be the enzyme that mediates down-regulation of D2 synthesis in the dark in this species.

To substantiate the idea that dark-grown cells contain an activity that reduces the Nac2/RBP40 disulfide bridge, we mixed stromal protein extracts from light- and dark-grown wild-type cells in a 1:1 ratio. When this mixture was assayed, a drastic decrease in the level of the Nac2/RBP40 complex was observed which cannot be explained by a dilution effect (Fig. 6b). In the presence of 250 µM NADPH, this effect was even more pronounced, strongly suggesting that dark-adapted chloroplasts from

C. reinhardtii contain an activity, probably the algal NTRC, which severs the link between Cys_{11} of RBP40 and Nac2, and thereby turns down *psbD* gene expression.

Discussion

We have previously postulated that the translational activator RBP40, together with an RNA stem loop structure encompassing the AUG start codon, form a molecular switch with the capacity to regulate chloroplast *psbD* gene expression (Klinkert et al. 2006). Here, we demonstrate that this molecular switch is indeed involved in controlling D2 synthesis in a light-dependent manner (Fig. 7). Suppressor lines in which the requirement for RBP40 is bypassed, exhibited reduced levels of light-induced D2 synthesis (Fig. 1b). This argues for a light-dependent resolution of the RNA structure by RBP40. We have previously shown that recruitment of RBP40 by the Nac2 complex specifies its interaction with the *psbD* 5´ UTR (Schwarz et al. 2007). Intriguingly, this recruitment process and the subsequent formation of a Nac2/RBP40 complex is light-dependent and, thus, most likely forms the critical step during dark/light transitions in patterns of *psbD* gene expression (Fig. 3b).

Moreover, the data reveal a direct interaction of RBP40 with Nac2 via a light-dependent disulfide bridge involving the single Cys residue in RBP40 at position 11, suggesting that the redox state of Cys_{11} is the main target for the light control mechanism. To date, no RBP40 knock-out mutant lines are available, which hampers site-directed genetic approaches to confirm the role of Cys_{11} *in vivo*. However, the redox state of Cys_{11} apparently has no direct influence on the RNA-binding activity of RBP40, which is consistent with the localization of Cys_{11} in the N-terminal segment of RBP40, relatively remote from its predicted RNA-binding domain which starts at position 39 (Barnes et al. 2004, Fig. 2a). This RNA-binding domain is made up of four conserved repeats, each spanning 70 amino acids, and is structurally related to other RNA-binding domains of the RBD or KH type (Barnes et al. 2004, Fig. 2a). In agreement with this, a truncated version of RBP40 lacking the first 18 N-terminal amino acids – including Cys_{11} – has been shown to retain general RNA-binding activity (Barnes et al. 2004).

The question arises as to which Cys residue in the Nac2 protein interacts with Cys_{11} of RBP40. Nac2 encodes a total of eleven Cys residues at various positions, some of which might form intramolecular disulfide bonds, as suggested by the 2D redox PAGE analyses (Fig. 4; suppl. Fig. 2). Interestingly, the most probable disulfide

bridge is predicted to be formed between Cys_{981} and Cys_{1008}, which are both located within the tetratricopeptide repeat (TPR) domain of Nac2. This domain has previously been shown to play a critical role for Nac2 function, probably by mediating the interaction with other subunits of the Nac2 complex (Boudreau et al. 2000). In addition, a putative dinucleotide-binding domain is predicted at position 402 – 413 of Nac2 which might be involved in the modulation of the redox state of one or more of its Cys residues. However, only a systematic evaluation of these sites can uncover the residue that forms the link with RBP40.

The most interesting question concerns the light-mediated redox switch at Cys_{11}. The current models for redox control of chloroplast gene expression usually involve light-catalyzed reduction processes which are linked to the photosynthetic electron transport (PET) via either PSI and thioredoxin or the redox state of the plastoquinone pool (Barnes and Mayfield 2003). In case of Nac2/RBP40 complex formation, PET is apparently not directly involved in the regulatory process since Cys_{11} oxidation is also observed in the PSII mutant *mbb1*. This is consistent with previously measured wild-type levels of D2 synthesis rates in this mutant (Vaistij et al. 2000). PSI deficiency leads to oxidative stress in the light, which obviously affects NAC2/RBP40 complex formation and results in a shutdown of *de novo* PSII synthesis, thereby avoiding harmful photosynthetic electron overflow. The reductive detachment of RBP40 from Nac2 under oxidative stress conditions is likely to be mediated via gluthationylation of RBP40 as has been reported previously (Michelet et al. 2008, Fig. 4c).

In the dark, however, a different regulatory redox system appears to operate on the Nac2/RBP40 disulfide bridge, namely the NTRC system. NTRC uses NADPH as source of reducing power, which can be produced during darkness by the oxidative pentose phosphate pathway (Neuhaus and Emes 2000). Thus it was proposed that NTRC allows redox regulation in the chloroplast during the night, a notion supported by the hypersensitivity of the Arabidopsis NTRC knock out mutant to prolonged darkness (Pérez-Ruiz et al. 2006). Recently the regulation of the ADP-Glc pyrophosphorylase (AGPase) involved in starch synthesis in *A. thaliana* was shown to be mediated by NTRC (Michalska et al. 2009). Our data suggest that this enzyme, which is active in the dark, is also involved the regulation of chloroplast *psbD* gene expression – at least in *C. reinhardtii*. Thus, NTRC would directly link the regulation of chloroplast gene expression to carbon metabolism in the chloroplast, i.e. the oxidative pentose phosphate pathway.

In conclusion, the following scenario is likely to describe the molecular events which underlie light-dependent regulation of D2 synthesis (Fig. 7). In the light, *psbD* mRNA translation is activated by RBP40 which is tightly bound via its Cys_{11} residue to Nac2, and is thereby targeted to its cognate binding site within the *psbD* 5´ UTR. The electron acceptor during formation of the disulfide bridge is not yet known. As discussed by Wittenberg and Danon (2008), reactive oxygen species, GSSG and O_2 might serve the purpose. RBP40 binding alters the RNA conformation at the initiation codon, making it accessible to the translation machinery. In the dark, reduction of the disulfide bond between Nac2 and RBP40 via NTRC leads to detachment of RBP40 from the Nac2 complex, resulting in down-regulation of D2 synthesis. Thus, the redox state of the Nac2/RBP40 disulfide bridge appears to represent the key control point for regulation of D2 synthesis, which – in light of the CES principle of PSII assembly – represents the key player in determining PSII levels in the green alga *C. reinhardtii* (Minai et al. 2006).

Methods

Strains and culture conditions

C. reinhardtii strains were grown in continuous light (30 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 23°C in Tris-acetate-phosphate medium containing 1% sorbitol (TAPS, Gorman and Levine 1965). For dark adaption, cells were transferred to complete darkness for 38 h prior to analysis.

Analysis of nucleic acids

Whole-cell RNA was prepared with TriReagent (Sigma-Aldrich) according to the manufacturer's instructions, and 2-µg aliquots were electrophoretically fractionated on gels, blotted onto positively charged nylon membranes and hybridized to *atpB* and *psbD* probes. Probes were generated by PCR using DIG-11-dUTP (Roche Diagnostics) and primers specific for the respective target genes (psbD: 5'-GTAATACGACTCACTATAGGGCCACAATGATTAAAATTAAA-3', 5'-GTTGGTGTCAACTTGGTGG-3' / atpB: 5'-ATGTTGTCCAGCGTGCGC-3', 5'-TTACTTCTTGGGCAGGAG-3'). Visualization of hybridization signals was performed by ECL using AP-conjugated anti-DIG-antibody and CDP* substrate (Roche Diagnostics).

Pulse labelling of proteins

Chlamydomonas liquid cultures were grown in TAPS medium to a density of ~ $2 \cdot 10^6$ cells/mL, pelleted, resuspended in TAPS medium in which all sulfur-containing ingredients were replaced by the respective chloride salts (TAPS-S), and incubated for 16 hours at 23°C in the light. Cells were pelleted, washed, and resuspended in TAPS -S/-T (lacking both sulfur salts and trace elements) and grown in the dark for 2 hours. Cells were then washed again and resuspended in TAPS -S/-T to a concentration of 80 µg chlorophyll per mL. Aliquots (225 µl) of the cell suspension were incubated with cycloheximide (10 µg/mL) for 10 minutes. Subsequently, 100 µCi $H_2{}^{35}SO_4$ (Hartmann Analytic, Braunschweig) was added to each, followed by incubation for 15 minutes in the light as before. After centrifugation, sedimented cells were frozen in liquid nitrogen. Cells were resuspended in 10 mM HEPES-KOH pH 7.5, 10 mM EDTA in the presence of CompleteMini protease inhibitors (Roche) and

disrupted by sonication (30 sec, RT). The homogenate was then centrifuged at 20000 g for 30 min. The pellet was resuspended in 10 mM HEPES-KOH pH 7.5, 10 mM EDTA. Samples were fractionated by electrophoresis on a 16% sodium dodecyl sulfate-polyacrylamide gel containing 8 M urea.

UV cross-linking of RNA to recombinant RBP40

For expression of recombinant RBP40 protein, the DNA sequence encoding amino acids 1-382 was PCR-amplified from a cDNA clone using the primer pair BamHI-RBP40 (5'- aaggatccATGCTGACCTTGAGACGTGC-3') and RB38-DN44revSalI (5'-ttgtcgacCTAGTAGCGGGCGCCC-3'), and inserted into the plasmid pQE30 (Qiagen) via *Bam*HI/*Sal*I restriction sites. Protein expression in *E. coli* M15 blue cells (Stratagene) was induced by addition of IPTG to a final concentration of 1 mM, followed by growth at 37°C for 3 h. The recombinant protein was purified according to the GE Healthcare protocol for purification of histidine-tagged recombinant proteins under native conditions using Ni-Sepharose 6 Fast Flow (GE Healthcare). In preparation for the binding reactions described below, the purified protein was incubated for 1 h at RT with 25 mM GSSG, 50 mM GSH, or a 50-fold molar excess of NEM, respectively, in a buffer containing 100 mM HEPES/KOH pH 7.8, 25 mM $MgCl_2$ and 300 mM KCl, followed by desalting using Amicon Ultra centrifugal filtration devices (Millipore) with a 10-kD molecular mass cutoff, in accordance with the manufacturer's instructions.

The DNA template for *in vitro* synthesis of the *psbD* RNA probe was generated by PCR using T7psbD5 (5'-gtaatacgactcactatagggCCACAATGATTAAAATTAAA-3'; T7 RNA polymerase promoter in lower case letters) and psbDUTR3 (5'-ACCGATCGCAATTGTCAT-3') as primers. RNA synthesis was catalyzed by T7 RNA polymerase (Fermentas) in the presence of [α-32P]UTP (3000 Ci/mmol; Hartmann Analytic), according to the manufacturer's protocol. After removal of the template by treatment with DNase I (Promega), the RNA was extracted with phenol-chloroform and precipitated with ethanol in the presence of ammonium. Binding reactions (20 µl) were performed at RT for 5 min and contained 500-1000 kcpm of ^{32}P-labeled RNA probe, 20 mM HEPES/KOH pH 7.8, 5 mM $MgCl_2$, 60 mM KCl, and 3 µg of pretreated protein. After irradiation, the free RNA probes were digested by treatment with 10 U RNase One (Promega) for 30 min at 37°C, and the samples were fractionated by SDS-PAGE, and analyzed by phosphorimaging.

Gel filtration analysis of native proteins

For analysis of native protein complexes, chloroplasts were isolated from *cw15* strains according to Zerges and Rochaix (1998), and lysed in non-reducing breaking buffer (10 mM EDTA, 10 mM Tricine-KOH pH 7.5, and Roche CompleteMini protease inhibitors). Membrane material was removed by centrifugation on a 1 M sucrose cushion (100000 g, 30 min). Reducing conditions, if indicated, were achieved by adding 5 mM GSH to the stroma-containing supernatant prior to concentration using Amicon Ultra filtration devices (Millipore). Samples (~2 mg protein) were loaded through an SW guard column onto a 2.15 × 30-cm G4000SW column (Tosoh), and elution was performed with gel filtration buffer (50 mM KCl, 5 mM $MgCl_2$, 5 mM □-aminocaproic acid, 20 mM Tricine-KOH pH 7.5), at a flow rate of 2 mL/min (Johnson et al. 2010). All steps were performed at 4 °C

Diagonal 2D redox SDS–PAGE

Stromal proteins (100□µg) from *cw15* strains were isolated according to Zerges and Rochaix (1998) in the absence of reducing agents. To prevent thiol reoxidation, proteins were alkylated with 0.1 M iodoacetamide in the dark (15 min, 4 °C). An appropriate volume of non-reducing Laemmli buffer was added, and the samples were separated by SDS-PAGE in the first dimension (10 % polyacrylamide) resolving gel. After electrophoresis, gel lanes were excised and incubated in SDS running buffer containing 0.1 M DTT (10□min, RT), before incubation with 0.1 M iodoacetamide in the same buffer (10 min, RT). The gel strips were then horizontally applied to another 10% SDS-PA gel, and electrophoresis was performed in the second dimension (Ströher and Dietz 2008; Stengel et al. 2009). Immunoblotting was carried out with antibodies raised against Nac2 and RBP40 (Schwarz et al. 2007).

PEGylation

Stromal proteins (20 µg) were treated with 10 mM methoxypolyethylenglycol-maleimide (5 kDa, PEG-MAL; Laysan) in alkylation buffer (0.1 M Tris-HCl pH 7.0, 1 mM EDTA) for 0, 10, 20 and 30 minutes, at 4 °C in the dark. The reaction was stopped by addition of Laemmli buffer in the presence of 0.1 M DTT (Balsera et al.

2009). Sauer:bis-Tris SDS-PAGE (10%) and MOPS running buffer were used to separate the proteins. RBP40 was detected by immunoblot analysis.

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Figure legends

Figure 1: Rates of D2 synthesis depends on light conditions.

(**a**) Thylakoid membrane proteins of indicated strains were pulse labeled with ^{35}S-sulfur, fractionated by SDS-PAGE in 16 % gels containing 8 M urea, and visualized by autoradiography. D: cells were grown in the dark for 38 h prior to analysis; L: cells were grown in continuous light at 30 µmol $m^{-2}s^{-1}$. (**b**) Densitometric quantification of D2 protein synthesis rates shown in (**a**). The results are representative of three independent experiments. D2 signals were normalized relative to the AtpA signal marked by the *star* in (**a**). The relative increase of D2 synthesis upon light induction is indicated.

(**c**) Northern blot analysis of *psbD* and *atpB* transcripts from the indicated strains grown under the same conditions as in (**a**).

Figure 2: Thiol labellingand RNA binding activity of RBP40.

(**a**) Schematic representation of the RBP40 polypeptide showing the disposition of the four repeats involved in RNA binding relative to the single Cys residue (Cys_{11}). The C-terminal end of the putative transit peptide predicted by the TargetP 1.1 algorithm (30 amino acids, http://www.cbs.dtu.dk/services/TargetP) is indicated by the scissors. (**b**) Time course of Cys_{11} alkylation. Stromal proteins (20 µg) were treated with 10 mM mPEG5000-maleimide for the periods indicated, and then subjected to immunoblot analysis with an anti-RBP40 antibody. (**c**) RNA-binding activity of full-length recombinant RBP40 protein (rRBP40). Aliquots (3 µg) of rRBP40 were pretreated with 25 mM GSSG, 50 mM GSH, or a 50-fold molar excess of NEM, UV-crosslinked to a radiolabelled *psbD* 5´UTR probe and fractionated by SDS-PAGE.

Figure 3: Light-dependent formation of Nac2/RBP40 complexes.

Wild-type (**a**) and mutant (**b**) cells were grown under the conditions indicated on the left margin (see Fig. 1 for details) and subjected to SEC. Fractioned proteins were subjected to Western blotting and labeled with the antibodies indicated on the right. The samples marked LR was treated with 5 mM gluthatione prior to SEC. Fraction numbers and molecular weights are indicated at the top.

Figure 4: Light-dependent formation of a disulfide bridge between Nac2 and RBP40. (**a**) Aliquots (100 µg) of stromal proteins from light-grown wild-type cells were fractionated by 2D redox SDS-PAGE, and Nac2 and RBP40 were localized by immunoblot analysis. The diagonal along which polypeptides that form no S-S bonds are expected to lie is indicated. For further explanation, see text. (**b**) Sections of 2D redox gels showing immunodetected Nac2 and RBP40 signals after 2D electrophoresis of samples from strains grown under the indicated conditions. The samples marked LR were reduced with 5 mM glutathione prior to electrophoresis in the first dimension. For details see Figs. 1 and 3.

Figure 5: Blue and red light do not affect Nac2/RBP40 disulfide bridge formation.
Stromal proteins were isolated from dark-adapted wild-type cells that had been exposed to low levels (5 µmol m^{-2} s^{-1}) of either blue (BL) or red light (RL) for 3 h, and subjected to 2D redox PAGE. Nac2 and RBP40 proteins were localized by immunoblot analysis.

Figure 6: The Nac2/RBP40 disulfide bridge can be reduced by rNTRC.
(**a**) Stromal proteins isolated from light-grown wild-type cells were incubated with 250 µM NADPH in the presence or absence of 2 µM rNTRC enzyme from *Anabena* sp. PCC 7120, and analyzed by 2D redox PAGE. The rNTRC enzyme was prepared as reported in Pascual et a. (2011).
(**b**) A 1:1 mixture of 50 µg each of stromal proteins from dark-adapted and light-grown cells was incubated in the presence or absence of 250 µM NADPH prior to 2D redox PAGE. Nac2 and RBP40 proteins were detected by immunoblot analysis.

Figure 7: Working model for redox regulation of *psbD* gene expression.
In the light, RBP40 binds to Nac2 via an intermolecular disulfide bridge and, as a consequence, the RNA conformation at the AUG start codon is altered. This allows ribosomes access to the initiation site and enables efficient translation of *psbD* mRNA. In the dark, the disulfide bridge between Nac2 and RBP40 is reduced via NTRC leading to detachment of RBP40 from Nac2 and down-regulation of D2 synthesis. For further explanation see text.

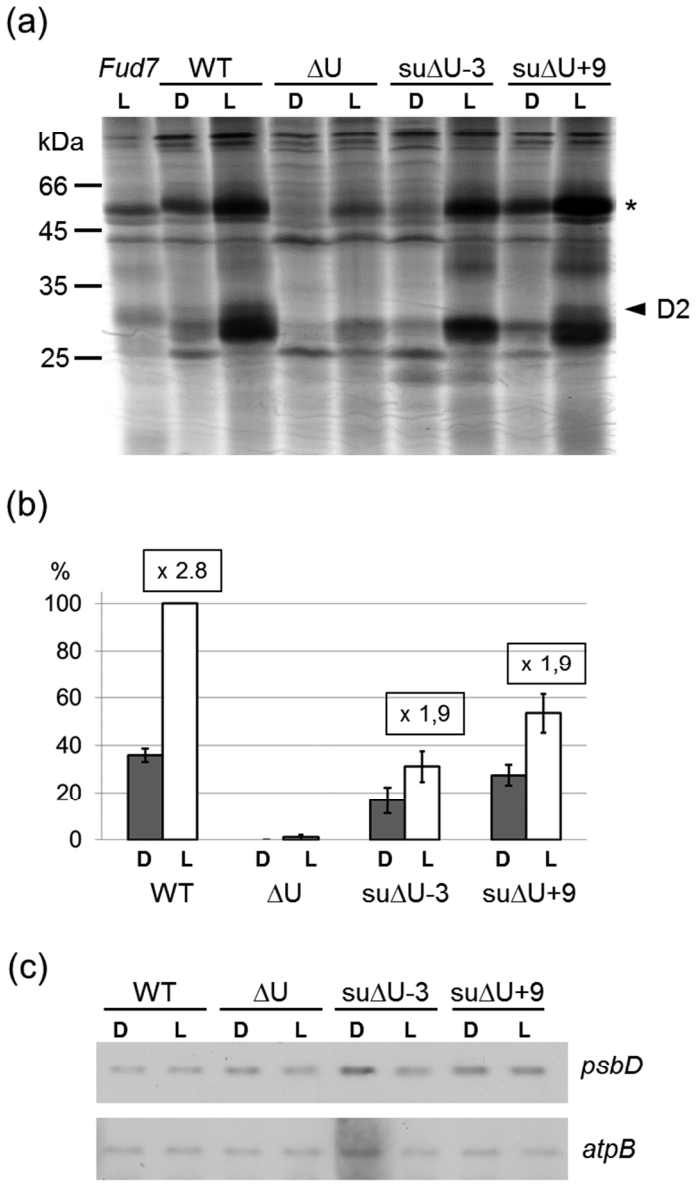

Figure 1
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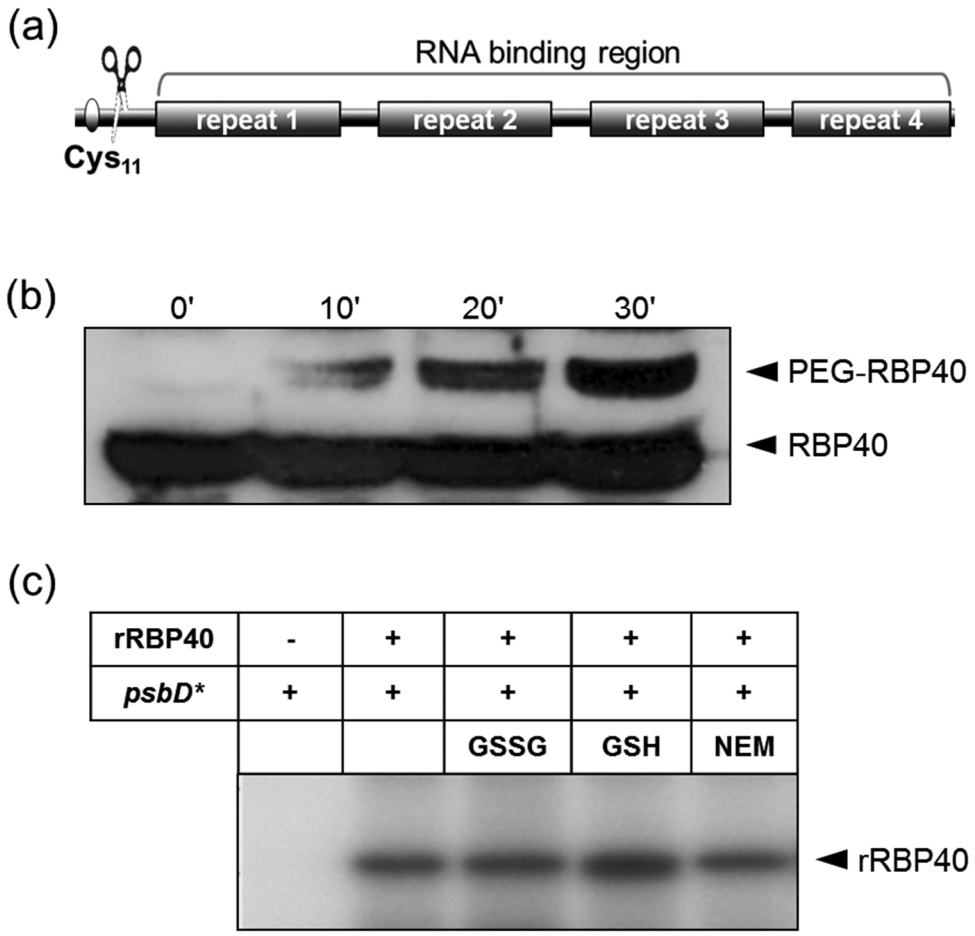

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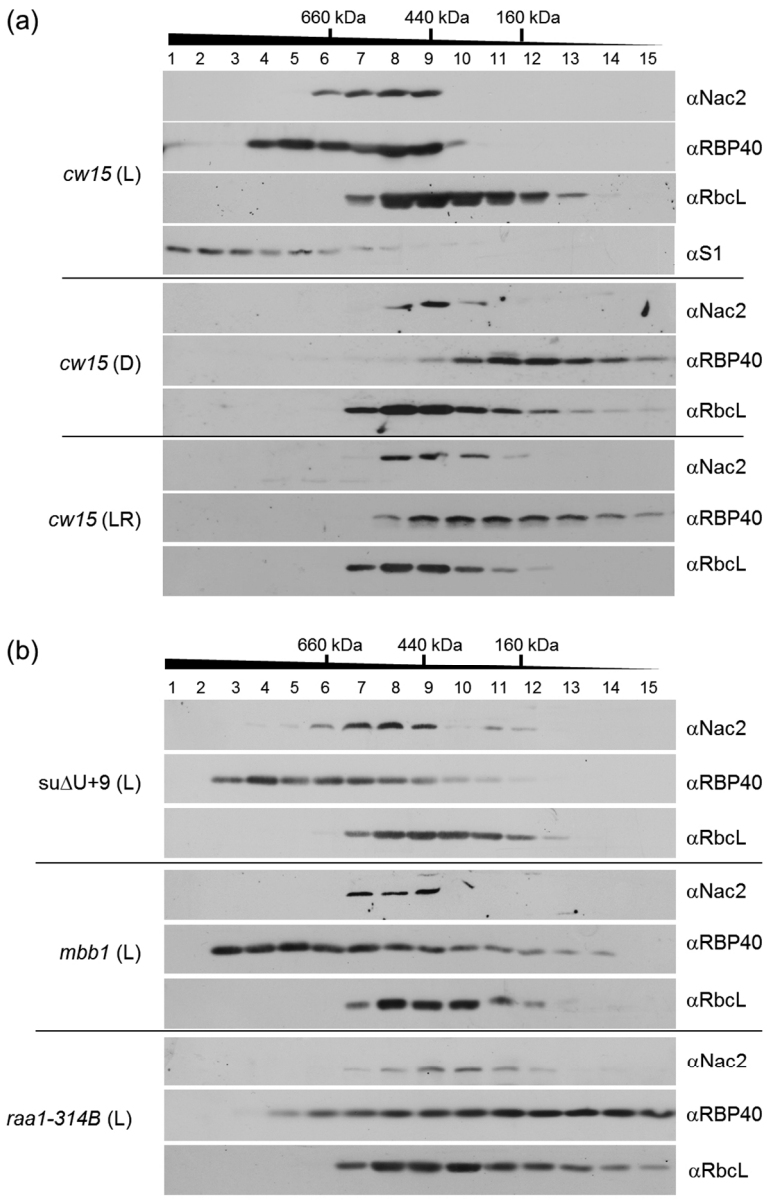

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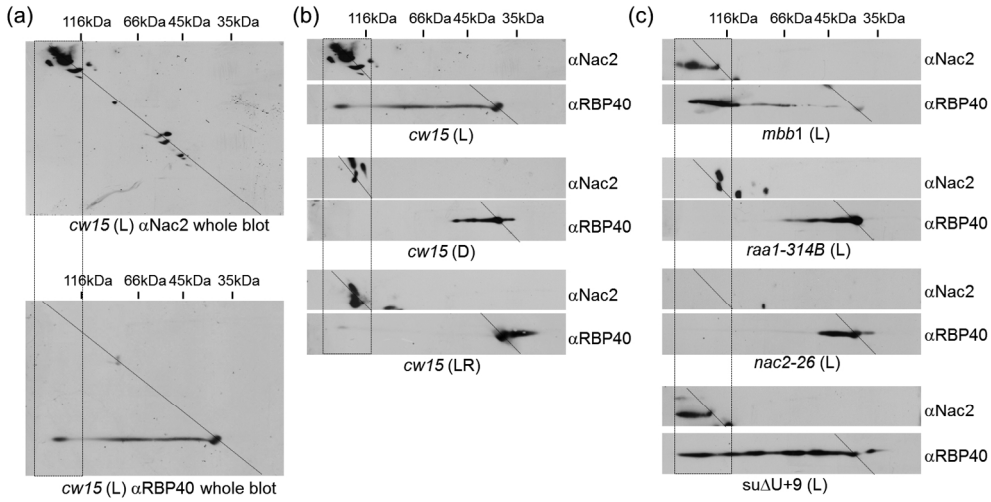

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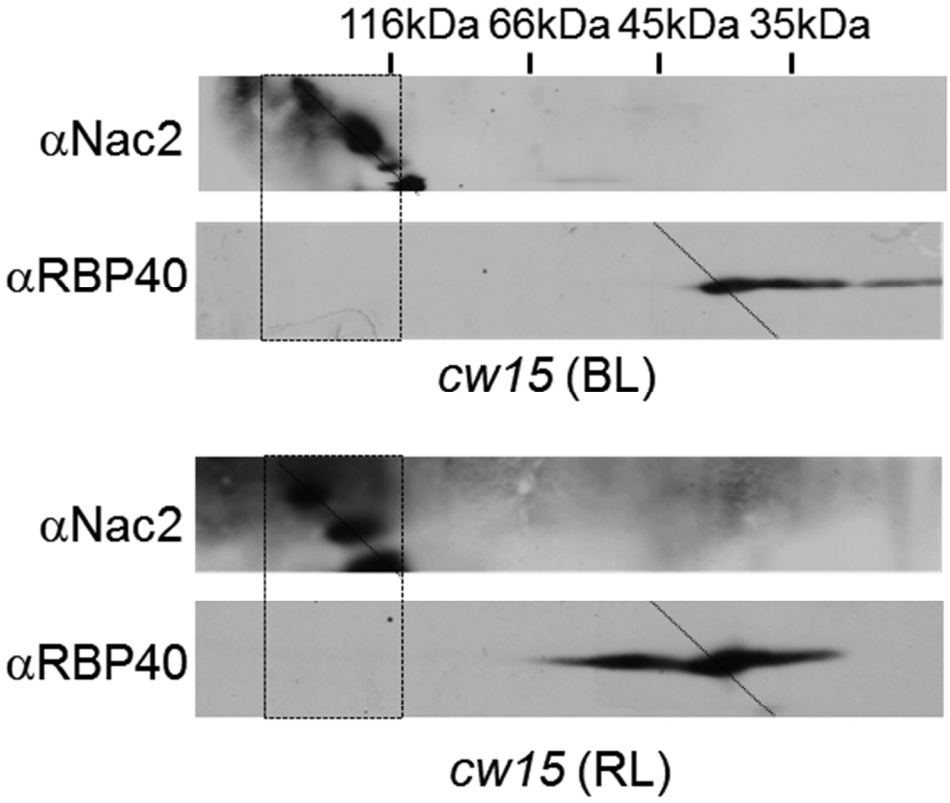

Figure 5
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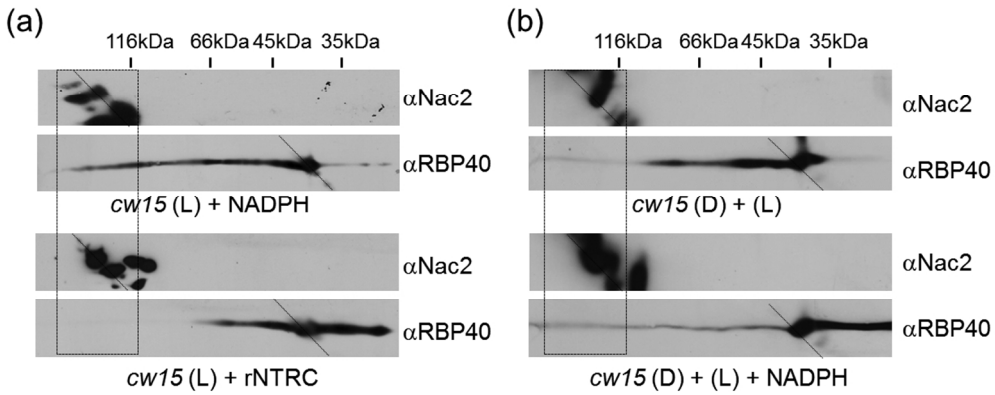

Figure 6
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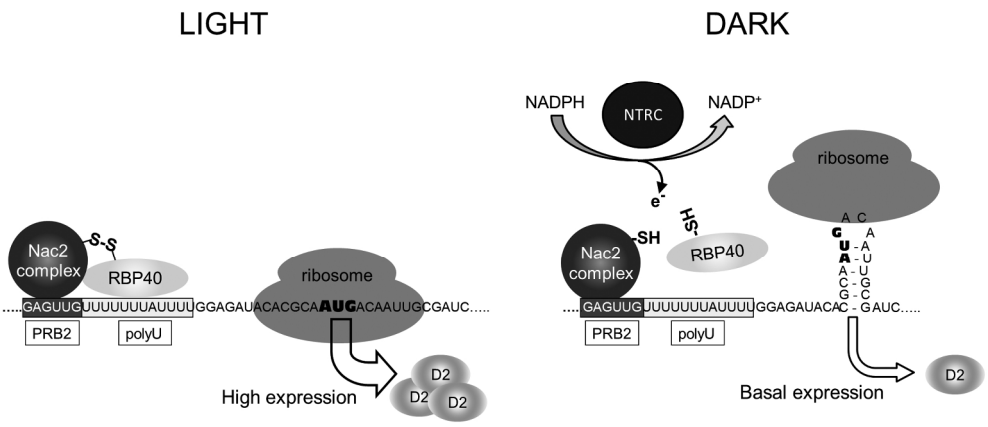

Figure 7
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3.4 Cysteine modification of a specific repressor protein controls the translational status of nucleus-encoded LHCII mRNAs in *Chlamydomonas*. (2009)

Wobbe, L., Blifernez, O., **Schwarz, C.**, Mussgnug, J.H., Nickelsen, J. and Kruse, O. *Proc. Natl. Acad. Sci. USA*, 106, 13290 – 13295

In this study, NAB1, a cytosolic RNA-binding protein, which is involved in translation of nuclear encoded PSII proteins in *C. reinhardtii* was analyzed in regard to its redox-dependent RNA binding ability. NAB1 is a translational repressor of LHCII mRNAs, preferentially binding to the *lhcbm6* isoform. It was established that the redox state of two cysteine residues in this protein regulates the RNA binding activity. Mutants lacking these residues had a small-antenna phenotype even under low-light conditions when cysteines were replaced by serines. The thiol groups of these cysteines are required in their reduced state for specific RNA binding. The color of the cultures already indicated the phenotype due to lower chlorophyll content in mutant cells. Polysomal analyses showed that decreased amounts of chlorophyll and antennae are caused by less efficient translation of *lhcbm6* transcripts. This was also confirmed by diminishing NAB1 binding affinity for its target RNA by alkylation of these cysteines. Additionally, binding capacity of mutated NAB1 was less prone to oxidative stress. It was shown by co-immunoprecipitation of RNA that the redox state of NAB1's cysteine residues directly affects the translation repressor activity of that protein *in vivo*. The repressor can be reversibly deactivated by modification of its cysteines and becomes fully active when those amino acid residues return to their dithiol state.

My contribution to this publication was the performance of *in vitro* RNA binding studies by UV crosslinking followed by SDS-PAGE separation of the recombinant NAB1 protein and labeled *lhcbm6* RNA as well as unlabeled *psbD* 5´ UTR under different redox conditions. Lutz Wobbe and Olga Blifernez did all the *in silico* and *in vivo* analyses, while Lutz Wobbe contributed the chemical agents and the analysis tools. The manuscript was written by Lutz Wobbe, Jan Mussgnug, Jörg Nickelsen, and Olaf Kruse with the final supervision by Olaf Kruse.

Cysteine modification of a specific repressor protein controls the translational status of nucleus-encoded LHCII mRNAs in *Chlamydomonas*

Lutz Wobbe[a], Olga Blifernez[a], Christian Schwarz[b], Jan H. Mussgnug[a], Jörg Nickelsen[b], and Olaf Kruse[a,1]

[a]Department of Biology, University of Bielefeld, Algae BioTech Group, Universitätsstrasse 25, D-33615 Bielefeld, Germany; and [b]Ludwig-Maxmilians-Universität München, Department Biologie I–Molekulare Pflanzenwissenschaften, Grosshaderner Strasse 2-4, D-82152 Planegg-Martinsried, Germany

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The cytosolic RNA-binding protein NAB1 represses translation of LHCII (light-harvesting complex of photosystem II) encoding mRNAs by sequestration into translationally silent mRNP complexes in the green alga *Chlamydomonas reinhardtii*. NAB1 contains 2 cysteine residues, Cys-181 and Cys-226, within its C-terminal RRM motif. Modification of these cysteines either by oxidation or by alkylation in vitro was accompanied by a decrease in RNA-binding affinity for the target mRNA sequence. To confirm the relevance of reversible NAB1 cysteine oxidation for the regulation of its activity in vivo, we replaced both cysteines with serines. All examined cysteine single and double mutants exhibited a reduced antenna at PSII caused by a perturbed NAB1 deactivation mechanism, with double mutations and Cys-226 single mutations causing a stronger and more distinctive phenotype compared with the Cys-181 mutation. Our data indicated that the responsible redox control mechanism is mediated by modification of single cysteines. Polysome analyses and RNA co-immunoprecipitation experiments demonstrated the interconnection of the NAB1 thiol state and its activity as a translation repressor in vivo. NAB1 is fully active in its dithiol state and is reversibly deactivated by modification of its cysteines. In summary, this work is an example that cytosolic translation of nucleus encoded photosynthetic genes is regulated via a reversible cysteine-based redox switch in a RNA-binding translation repressor protein.

Chlamydomonas reinhardtii | light harvesting antenna | redox control | translation control

To compensate for changes in light intensity or spectral quality, plants have developed several short-term and long-term mechanisms to regulate the amount of light that is captured by each photosystem (1). One important long-term adaptation strategy of plant organisms involves the complex expression regulation of various nuclear-encoded light harvesting complex (*Lhcb*) genes (1). All levels of LHCII gene expression are targeted by regulation mechanisms (2–5) which rely on a complex retrograde and anterograde communication between plastid, nucleus, and cytosol (6). The cytosolic translation repressor NAB1, which was identified in a *Chlamydomonas reinhardtii* light acclimation mutant (4), is the center of interest within this work. NAB1 harbors 2 RNA-binding motifs and 1 of these motifs, located at the N terminus, belongs to the highly conserved family of CSD (cold shock domain) domains. Proteins containing a CSD motif are referred to as Y-box proteins and eukaryotic members of this large family generally contain a second auxiliary RNA-binding domain, which modulates the RNA affinity of the protein but can be dispensable for selective RNA recognition (7). In the case of NAB1, the CSD motif is combined with a C-terminal RRM (RNA recognition motif) domain, which was demonstrated not to be essential for selective RNA recognition (4). It was shown that NAB1 binds to the mRNA of LHCBM (major light-harvesting complex of photosystem II) genes, thereby preventing translation via sequestration of the message in translationally silent messenger ribonucleoprotein complexes (mRNPs). The LHCII complex of *C. reinhardtii* is constituted by 10 individual highly homologous LHCBM isoforms (8, 9), and NAB1 displays selectivity toward distinct isoforms with *LHCBM6* mRNA being 1 of its main targets (4). It has been shown for numerous proteins that reversible modification of cysteine residues can act as an effective activity switch (10). In this work, we intended to investigate whether the composition of the light-harvesting antenna of PSII is controlled via the redox state of 2 cysteines, which are located in the C-terminal RRM domain of NAB1.

Results

Free Cysteines Are Required for Full RNA-Binding Activity of NAB1 in Vitro. NAB1 harbors 2 cysteine residues, located at amino acid positions 181 and 226 within the C-terminal RRM domain. A structural model of the RRM domain of NAB1 was generated using the NMR structure of the highly homologous RRM motif of human RNA binding protein hnRNP M (Fig. 1*A*). Within this structure Cys-181 is part of a loop structure whereas Cys-226 is part of the α-helix α_2 and both residues are separated by 14.97 Å. Exposition of these cysteines on the protein surface is a prerequisite for a potential reversible interaction with thiol modifying compounds in vivo. Modeling of the C terminus (Fig. S1) indicated that Cys-181 is buried in a groove-like structure together with 2 leucine residues and surrounded by uncharged amino acids creating an environment of low electrostatic potential. In contrast, Cys-226 could be more reactive because it is positioned in an exposed surface area at the interface of a negatively and positively charged patch. To analyze whether modification of cysteines within the RRM motif has an impact on the binding affinity toward its cellular mRNA target *LHCBM6*, RNA-binding studies with oxidized and reduced recombinant NAB1 were performed (Fig. 1*B*). For these experiments, a probe containing the CSDCS (cold shock domain consensus motif) motif of *LHCBM6* was chosen, which was previously shown to bind NAB1 specifically (4). Because of the reducing conditions used for NAB1 purification, recombinant NAB1 proteins were maintained in a reduced state. Under this condition they efficiently bound a radioactive CSDCS probe derived from *LHCBM6* (Fig. 1*B*, untreated). The presence of unlabeled competitor RNA (Fig. 1*B*, *psbD* +) had a negligible effect on binding efficiency, indicating sequence specificity of the protein-RNA interaction (4). In contrast, when shifted to an oxidized form by treatment with glutathione disulfide (GSSG),

Author contributions: L.W., J.H.M., J.N., and O.K. designed research; L.W., O.B., and C.S. performed research; L.W. contributed new reagents/analytic tools; L.W., O.B., C.S., J.H.M., J.N., and O.K. analyzed data; and L.W., J.H.M., J.N., and O.K. wrote the paper.

The authors declare no conflict of interest.

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[1]To whom correspondence should be addressed. E-mail: olaf.kruse@uni-bielefeld.de.

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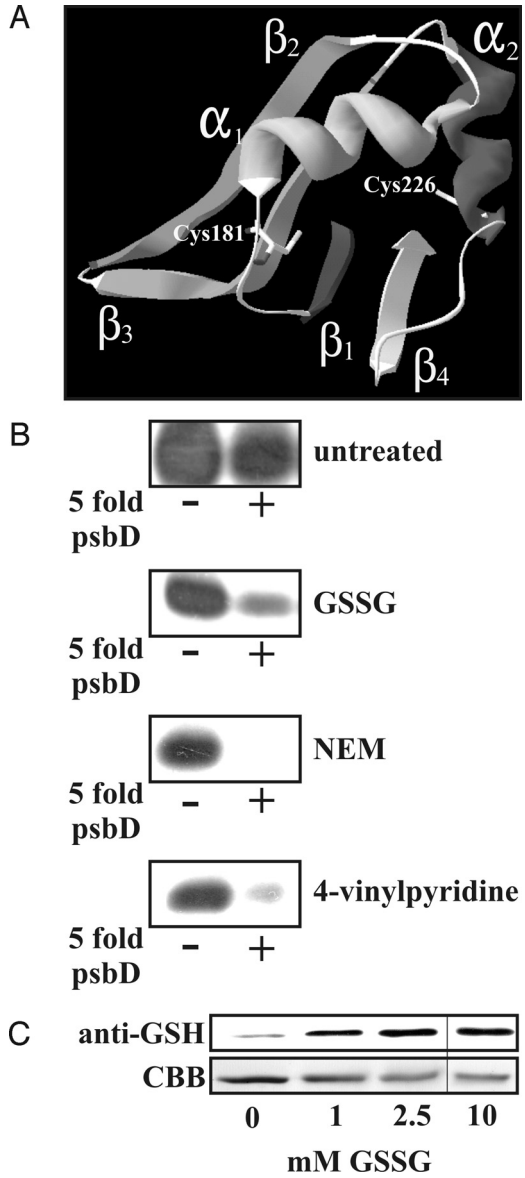

Fig. 1. In silico and in vitro analyses to examine a potential cysteine regulation of NAB1. (*A*) Homology model of the C-terminal RRM domain of NAB1. Cys-181 and Cys-226 within the RRM domain are indicated. (*B*) In vitro RNA binding studies. Autoradiogram of UV-cross-linked and SDS/PAGE separated NAB1-*LHCBM6*-CSDCS complexes. Recombinant NAB1 was either left untreated (*Top*) or oxidized with 5 mM glutathione disulfide (GSSG, *Middle*) before addition of a radioactively labeled *LHCBM6*-CSDCS probe alone (−) or of a mixture containing labeled *LHCBM6*-CSDCS and a 5-fold molar excess of unlabeled *psbD* competitor RNA (+). N-ethylmaleimide (NEM, third panel from *Top)* or 4-vinylpyridine (4-vinylpyridine, *Bottom*) was applied for cysteine alkylation. (*C*) Treatment of recombinant NAB1 with different concentrations of glutathione disulphide (GSSG) and immunodetection of protein-glutathione adducts (*Upper*). Coomassie blue (CBB) stain of recombinant NAB1 after treatment with the indicated GSSG concentrations (*Lower*).

the binding signal of the NAB1-RNA complex was most strongly reduced after addition of competitor RNA (Fig. 1*B*, GSSG, *psbD* +), showing that oxidation mainly affects the specific binding activity of NAB1. To investigate whether oxidation of both cysteines via the formation of an intramolecular disulfide is crucial for the observed decrease in RNA affinity, thiol-alkylating compounds were used to modify NAB1 cysteines individually. The thiol-specific alkylator N-ethylmaleimide (NEM) and 4-vinylpyridine turned out to be potent inhibitors of the specific binding activity of NAB1, which was indicated by a complete absence of *LHCBM6*-NAB1 complexes after competitor addition (Fig. 1*B*). This result indicated that modification of RRM cysteines impairs the specific binding of NAB1 to *LHCBM6* mRNA. An additional result was that intramolecular disulfide bridge formation is not a prerequisite for NAB1 deactivation in vitro. In line with these findings was that the treatment of recombinant NAB1 with GSSG resulted in the glutathionylation of single cysteines (Fig. 1*C*). To address the potential of intramolecular disulfide formation within NAB1 more precisely, a peptide mapping analysis with recombinant wild-type Wt-NAB1 and a recombinant Cys226Ser mutant was performed. Wt- and Cys-226-NAB1 protein was oxidized with diamide and subsequently digested by V8 protease treatment. Any intramolecular disulfide bridge formation in the Wt protein should result in an altered peptide fragment pattern compared with the corresponding Cys226Ser sample, for which intramolecular disulfide formation is not possible. However, nearly identical digestion patterns of Wt and Cys226Ser samples were obtained (Fig. S2). As an additional control, 1 part of each oxidized sample was rereduced with DTT before SDS/PAGE. After rereduction, the banding pattern remained unchanged, indicating again that no disulfide linked peptides were present in the oxidized digest samples. In summary, these results demonstrated that modification of individual cysteines impairs the activity of NAB1 in vitro and led us to investigate the in vivo relevance of thiol regulation as a mechanism to control the activity state of NAB1.

Replacement of RRM Domain Cysteines with Serine Creates a Small Antenna Phenotype. To determine whether the 2 cysteine residues have an essential regulative function in vivo, a mutagenesis strategy was applied. For this experiment, we selected a derivative strain of *Stm3* that expresses an HA-epitope-tagged form of the LHCBM6 protein. The HA-tagged LHCBM6 isoform is expressed under the control of the *PSAD* promoter (4, 11), thus decoupling its transcription from LHC promoter activity. This NAB1-free strain was transformed with a mutated version of the *NAB1* gene. This experimental setup allowed the direct correlation between NAB1 activity and post-transcriptional regulation of LHC isoform LHCBM6. Mutated constructs for each single cysteine (NSM) and for both cysteines (NDM) were obtained by site-directed mutagenesis which resulted in the exchange of cysteine with serine residues. After cotransformation of these constructs, 15 strains were successfully identified which stably expressed a mutated version of NAB1, carrying either the double mutation Cys-181/226Ser or the single mutations Cys181Ser and Cys226Ser. For precise chlorophyll analyses to investigate the relevance of both cysteines for LHC antenna size regulation (Fig. 2*A*), a control strain (C) was obtained by transformation of *Stm3* with a Wt version of *NAB1*, which is expressed under control of the same promoter that drives expression of mutated NAB1 in the cysteine mutants. Based on this control strain, the Cys double mutants and the single Cys226Ser mutants showed a similar phenotype with an increase of Chl *a*/*b* ratios [from 2.2 in the control strain (C) to ≈2.6/2.8 in the mutants] accompanied by a 15–25% decrease of total chlorophyll content (Fig. 2*A*). Because Chl *b* is exclusively bound by light-harvesting proteins whereas Chl *a* is also present in the PS core complexes, the Chl. *a*/*b* ratio provides a parameter for the overall LHC antenna size compared with the entire PSI and PSII complexes. Consequently, the increased Chl *a*/*b* ratios are indicative for smaller light-harvesting antenna systems due to a regulatory dysfunction of NAB1. Of particular note was however, that the Cys-181 mutants exhibited a less distinctive phenotype with no differences in total chlorophyll content indicating functional differences between Cys-181 and Cys-226.

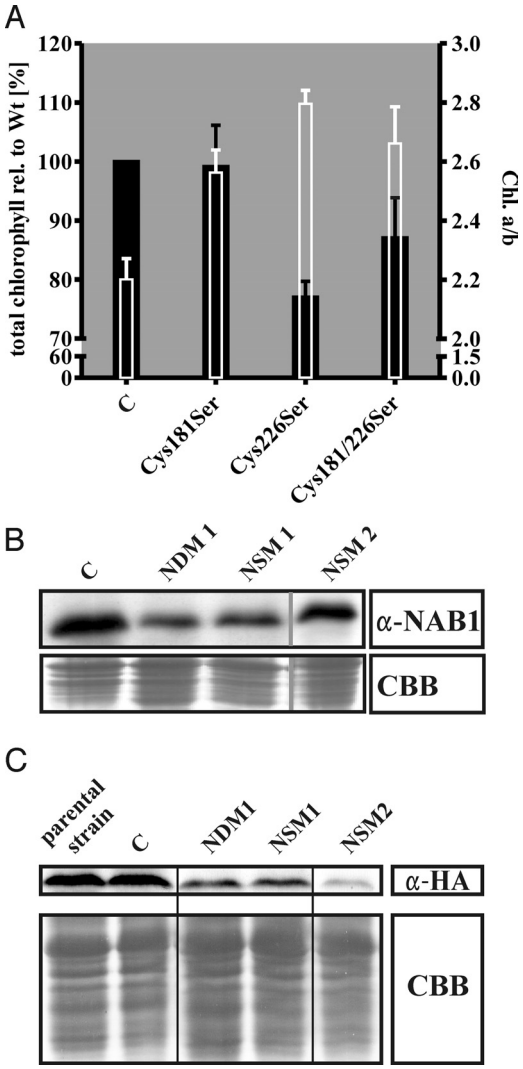

Fig. 2. Phenotypical analyses of NAB1-cysteine mutants. (*A*) Total chlorophyll content of NAB1-cysteine mutants relative to the control strain *C* expressing Wt-NAB1 (left *y* axis; black bars) and Chl. *a*/*b* ratios of cysteine mutants and control strain (right *y* axis; white bars). The data represent mean values of three independent chlorophyll measurements (using triplicates) performed with different strains for each cysteine mutation (10 strains expressing $NAB1_{Cys181Ser}$; three strains expressing $NAB1_{Cys226Ser}$; two strains expressing $NAB1_{Cys\text{-}181/226Ser}$). Error bars indicate standard deviations ($n = 30$ for $NAB1_{Cys181Ser}$; $n = 9$ for $NAB1_{Cys226Ser}$; $n = 6$ for $NAB1_{Cys\text{-}181/226Ser}$). (*B*) Anti-NAB1 immunoblot analyses to assess the NAB1 expression level in the Wt control strain and the cysteine mutant strains. (*Upper*) Representative immunoblot. (*Lower*) Coomassie blue stain (loading control). (*C*) Anti-HA-tag immunoblots to determine the expression of HA-epitope tagged LHCBM6 protein (*Upper*). (*Lower*) Coomassie blue-stained SDS protein gel (loading control).

To allow for more detailed analyses regarding the phenotypical characteristics caused by the mutations, we selected 1 representative strain for each single (NSM1: Cys181Ser; NSM2: Cys226Ser) and the double mutation (NDM1: Cys-181/226Ser). Immunoblot studies using a NAB1-specific antiserum demonstrated that the expression of NAB1 variants in these mutant strains was lower compared with the expression of Wt NAB1 in the control strain (Fig. 2*B*), excluding the risk that the significant decrease of antenna size was caused by increased levels of NAB1 protein.

Protein Expression of the Target mRNA *LHCBM6* Is Down-Regulated by Cysteine Mutation. Because all mutant strains expressed an HA-tagged version of the isoform LHCBM6, a comparative analysis of HA-LHCBM6 expression in the cysteine mutants, the parental strain, and in the control strain was a suitable method to analyze the effects of NAB1 cysteine mutation on its activity as a translation repressor in vivo. Transformation of the parental strain with mutagenized versions of *NAB*1, which lack 1 or both cysteines, yielded reduced HA-LHCBM6 amounts (Fig. 2*C*). Importantly HA-LHCBM6 expression of the mutant cell lines was also reduced compared with a cell line expressing wild-type NAB1 [Fig. 2*C*, lane 2 (C)].

Cysteine Mutants Are Unable to Enlarge Their LHCII Complexes Resulting in Impaired Growth Under Phototrophic Dim Light Conditions. Chlorophyll measurements and LHCBM6 protein expression studies indicated that the presence of cysteine residues within its RRM domain is crucial for a deactivation of NAB1. Photoautotrophic growth experiments in minimal medium (HSM) under dim light (40 μmol m^{-2} s^{-1}) were performed which force Wt *Chlamydomonas* cells to increase their light-harvesting antenna size to enhance the capture of photons for photosynthesis. All selected cysteine mutants showed a reduced growth rate under limiting light conditions when compared with the control strain (Fig. 3*A*). The exponential growth rates of the examined mutants varied between 0.0048Δ $O.D._{750\ nm}$/h in the case of NDM1, 0.0058Δ $O.D._{750\ nm}$/h for NSM1, and 0.0043Δ $O.D._{750\ nm}$/h for NSM2 and were therefore significantly reduced in relation to the control strain (0.0095Δ $O.D._{750\ nm}$/h). In good agreement with the results obtained under standard light conditions (see Fig. 2*A*), the Chl *a*/*b* ratio was highly increased in NDM1 and NSM2 only (2.24 ± 0.03 SD in control strain vs. 2.87 ± 0.03 SD in NDM1 and 3.09 ± 0.03 SD in NSM2), whereas the Cys-181 mutant NSM1 showed an increase to a much lesser extent (Chl *a*/*b* 2.32 ± 0.03 SD). In addition, total chlorophyll values in relation to the control strain (C) were only reduced in the double mutant NDM1 and strain NSM2 (76 ± 1% SD of control strain in case of NDM1 and 68 ± 2% SD for NSM2). Anti LHCII immunoblot studies (Fig. 3 *B* and *C*) confirmed that the amount of LHCII proteins was reduced in the cysteine mutants (72.8 ± 7.1% SE in the case of NDM1, 85.5 ± 12.3% SE for NSM1 and 67.7 ± 6.8% SE for NSM2 with the control strain being set to 100%) and demonstrated that the Cys-226 mutation again has a more severe effect on the LHC antenna size compared with the Cys-181 mutation (Fig. 3*C*). In conclusion, the observed phenotypes strongly indicate a direct correlation between the thiol state of the cysteines of NAB1 and the activity as a LHC translation repressor.

Cys:Ser Replacement in NAB1 Prevents the Deactivation of LHCBM Translation Repression. Polysome analyses were performed to investigate whether the observed reduced LHCII protein expression was caused by altered *LHCBM6* mRNA translation efficiency (Fig. 4). Sucrose gradient fractionation of cytosolic extracts was performed to separate nontranslated subpolysomal mRNPs, monosomal and polysomal complexes (4). According to our previous findings regarding the prominent role of Cys-226 for NAB1 regulation we selected the Cys226Ser mutation strain NSM2 for this experiment. The results presented in Fig. 4 demonstrated that the distribution of *LHCBM6* mRNA within the sucrose gradient of NSM2 was considerably different compared with the control strain. NSM2 displayed a high *LHCBM6* content exclusively in subpolysomal nontranslated RNA fractions (Fig. 4, fractions 1–4) and only low amounts in monosomal/polysomal fractions (Fig. 4, fractions 5 and greater) whereas the control strain showed high amounts of *LHCBM6* mRNA also in efficiently translated polysomal fractions. This result strongly indicated that the Cys-226 mutation causes an increased *LHCBM6* mRNA sequestration, which is in good accordance to the observed reduced HA-LHCBM6 and LHCII expression levels in vivo (Figs. 2*C* and 3 *B* and *C*).

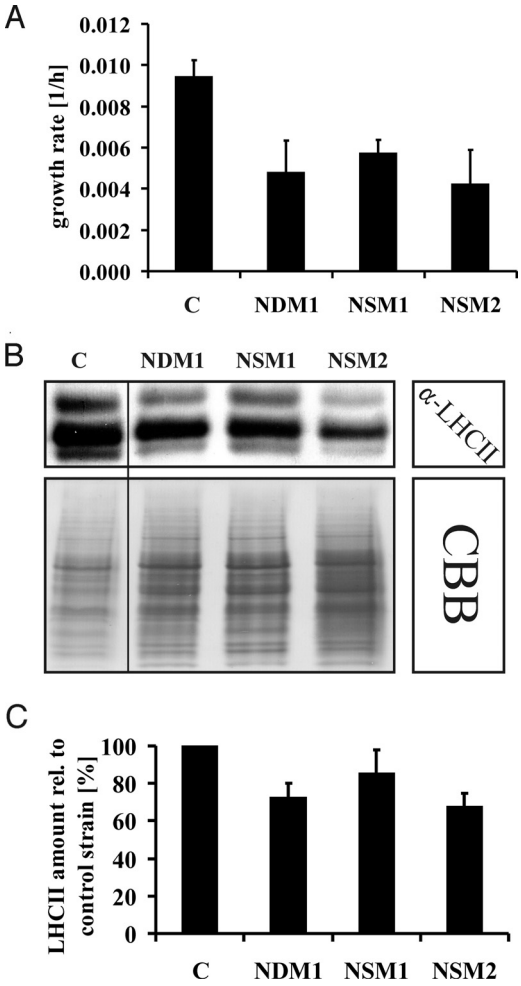

Fig. 3. Growth and photosynthetic low light acclimation of control strain (C) and NAB1 cysteine mutants. (*A*) Growth rates within the exponential phase observed under phototrophic low light conditions (HSM medium; 40 μmol m^{-2} s^{-1}). The growth rate was determined by measurements of the increase of the optical density per hour. Error bars indicate the standard deviation of three independent growth experiments. (*B*) Representative immunoblot using a LHCII-specific antiserum and Coomassie blue-stained SDS/PAGE gel. Protein samples were taken from cells grown under photoautotrophic dim light conditions (HSM medium; 40 μmol m^{-2} s^{-1}). (*C*) Results from three independent LHCII immunoblot analyses after phototrophic growth (HSM medium; 40 μmol m^{-2} s^{-1}) using samples of the control strain and the cysteine mutants. Signal intensities were quantified by densitometry and used to calculate the mean values represented by black bars. The amount of LHCII protein in the control strain was set to 100%. Standard errors are indicated by error bars.

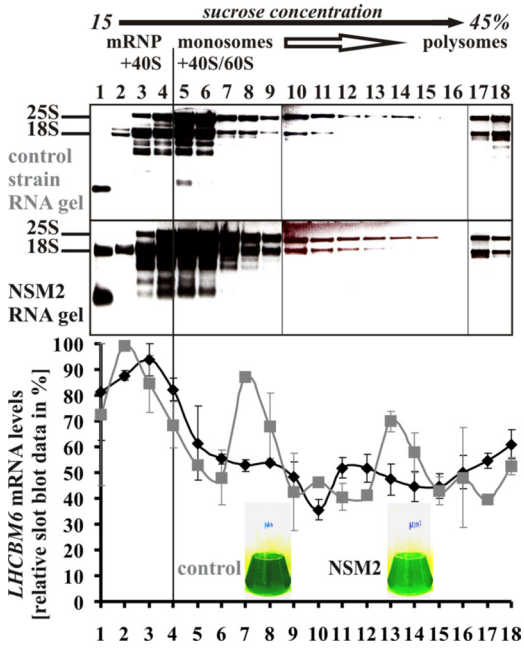

Fig. 4. Polysome analysis of control strain and NSM2 cells grown phototrophically under dim light conditions. Cytosolic extracts were centrifuged through a 15–45% continuous sucrose gradient to separate subpolysomal mRNPs, monosomes and polysomes. RNA was extracted from 18 gradient fractions and analyzed by formaldehyde-agarose gel electrophoresis and ethidium bromide staining. The amount of *LHCBM6* and *β-ACTIN* mRNA in each fraction was assessed by Northern slot-blot analysis. Slot-blot signals of *LHCBM6* were quantified by densitometrical scanning and normalized to the corresponding ß-*ACTIN* signal. The strongest *LHCBM6* blot signal obtained for each strain was set to 100%. Standard errors are based on three independent polysome fractionations.

Cys:Ser Mutation Results in a Higher Stability of NAB1-*LHCBM6* Complexes Under Oxidative Stress Conditions in Vivo. Characterization of the cysteine mutants strongly indicated that mutation of RRM cysteines perturbs the in vivo deactivation mechanism of NAB1. Consequently, we investigated whether oxidation of NAB1 changes its binding efficiency toward the target mRNA *LHCBM6* in vivo by RNA coimmunoprecipitation (4). Control strain, NSM1 (Cys181Ser), and NSM2 (Cys226Ser) cultures were grown and cysteine oxidation of NAB1 was induced by the addition of diamide. To follow the in vivo thiol state of both NAB1 cysteines we applied the thiol alkylating compound mPEG-MAL. mPEG-MAL exclusively reacts with the SH-group of free cysteines and enables to determine the number of free cysteines present in a protein at a certain time. Because protein modification by mPEG-MAL results in a large shift in the electrophoretic mobility, the number of free cysteines is directly correlated to the apparent molecular weight and can be traced via immunodetection (12). Diamide addition resulted in a strong shift from the reduced to the oxidized NAB1 thiol state (Fig. 5*A*). After 120 min of incubation the bulk of NAB1 was shifted to a fully oxidized state, containing no reduced cysteines. This time point was chosen to analyze the RNA-binding activity of fully oxidized NAB1 compared with the reduced NAB1 thiol states found in the cell under normal, stress-free conditions. Cysteine oxidation resulted in a strong reduction of RNA-binding affinity in the control strain to 19 ± 2% and in the Cys-181 mutant NSM1 (28 ± 5%) compared with the reduced state (Fig. 5*B*). In the case of the Cys-226 mutant NSM2 however, more than half of the binding activity remained (57 ± 1%) demonstrating a higher resistance of $NAB1_{Cys226Ser}$ toward oxidative deactivation (Fig. 5*B*). Importantly, the amount of NAB1 was not significantly affected by the diamide treatment in all examined mutants and the control strain (Fig. S3). The results demonstrated that the oxidation of NAB1 cysteines in vivo is accompanied by a decreased binding toward the *LHCBM6* target mRNA. The exchange of Cys-226 with serine strongly attenuates oxidative NAB1 deactivation, confirming that Cys-226 has a key function in the redox dependent activation of NAB1.

Discussion

The aim of the present study was to evaluate the possible relevance of 2 cysteine residues located in the RRM domain of NAB1 as central elements of an in vivo redox control mechanism, which determines its translation repressor activity. Preliminary studies carried out in vitro gave initial indications for the importance of the cysteines in controlling NAB1 activity,

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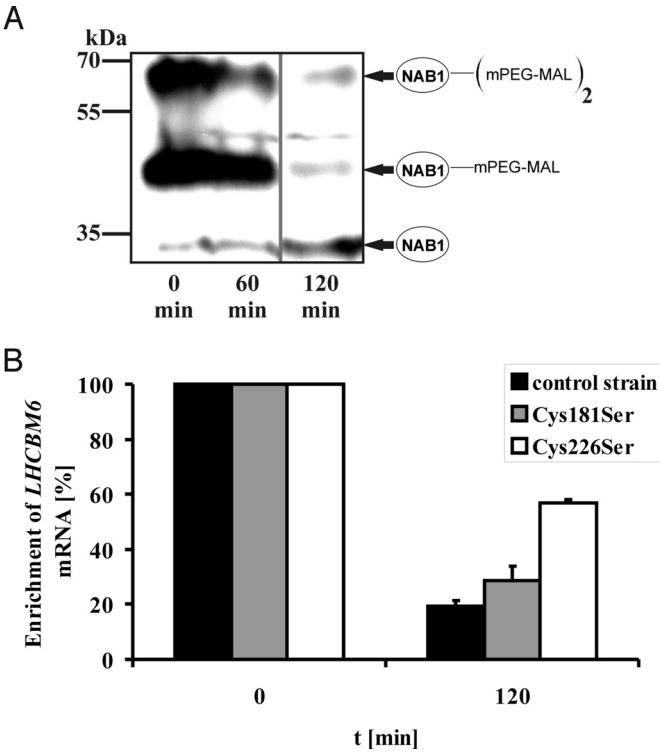

Fig. 5. Effects of cysteine oxidation on the RNA-binding capacity of Wt-NAB1 (control strain), $NAB1_{Cys181Ser}$, and $NAB1_{Cys226Ser}$ analyzed in vivo. (*A*) Examination of the Wt-NAB1 thiol state after 60 and 120 min after diamide addition (2 mM) to a liquid cell culture. The degree of cysteine modification was assessed by mPEG-MAL-labeling and subsequent anti-NAB1 immunoblot detection. (*B*) Coimmunoprecipitation of *LHCBM6*-mRNA using a NAB1-specific antiserum before and after diamide-induced oxidation of Wt -NAB1 (control strain), NSM1 (Cys181Ser) and NSM2 (Cys226Ser). The amount of coprecipitated *LHCBM6*-mRNA was quantified by RT-Q-PCR and the t_0-value was set to 100% for each strain. Error bars indicate the standard error of four independent RT-Q-PCR measurements.

which were subsequently confirmed by intense in vivo studies. In vitro studies indicated that full RNA-binding activity of NAB1 requires cysteines in their SH-states (Fig. 1*B*) and that cysteine modification by either glutathionylation or alkylation inhibits the specific RNA-binding activity of NAB1 (Fig. 1 *B* and *C*). Peptide mapping analyses of oxidized and reduced protein samples of Wt-NAB1 and a Cys226Ser mutant did not reveal the existence of disulfide linked peptides, challenging the importance of intramolecular disulfide formation for NAB1 redox control (Fig. S2). Replacement of both cysteines with serine yielded in a distinct phenotype characterized by a perturbed expression of LHCII proteins thus proving that the cysteines are crucial for NAB1 regulation in vivo (Figs. 2 and 3). These findings strongly indicated that NAB1 was arrested in a permanently active repressor state after both cysteines were replaced by serine. Further in vivo analyses, however, demonstrated that the single cysteine mutants $NAB1_{Cys181Ser}$ and $NAB1_{Cys226Ser}$ displayed clear different phenotypical characteristics (Figs. 2, 3*C*, and 5). The phenotypes of $NAB1_{Cys\text{-}181/226Ser}$ double mutants and the $NAB1_{Cys226Ser}$ single mutants were very similar, which makes it feasible to suggest that modification of Cys-226, if compared with modification of Cys-181, has a larger impact on the activity state of NAB1 in vivo (Figs. 2, 3*C*, and 5). It should be noted, however, that oxidative treatment of a mutant recombinant $NAB1_{Cys226Ser}$ protein caused a significant decrease in its in vitro *LHCBM6* RNA binding activity. This clearly demonstrates that Cys-181 is indeed involved in the deactivation of NAB1, although the phenotype of the corresponding Cys181Ser mutant cell lines is comparably milder than those of the Cys226Ser mutation. Finally polysome analyses together with mRNA-Coimmunoprecipitation studies (Figs. 4 and 5) fully demonstrated that in vivo deactivation of *LHCBM6* mRNA sequestration and accordingly translation repression depends on cysteine modification of NAB1. As a final conclusion from the sum of our in vivo results the translation repressor activity of NAB1 is determined by the thiol state of 2 cysteines located in the RRM domain. Oxidized cysteines represent the *off* state of the repressor, whereas reduced cysteines represent the *on* state. It has already been shown that RRM containing proteins from plant organisms involved in translational regulation of photosynthetic genes can be activity-regulated via cysteine modification (13). However, these proteins were shown to be located in the plastid. NAB1 represents a eukaryotic example of a cytosolic RRM protein being subject to cysteine-based redox control. Apart from *C. reinhardtii*, NAB1 analogous proteins containing a combination of CSD and RRM domains were only identified in the genomes of closely related algal species *C. incerta* (14) and *Volvox carteri* (15). The position of both cysteines is conserved in all 3 genome sequences indicating that the mechanism of redox regulation is conserved at least within the *Volvocales* taxonomic group of green algae. The cysteine residues of cytosolic proteins are maintained in the reduced thiol state by action of thiol-based redox buffer systems (glutathione/glutaredoxins; thioredoxins/thioredoxin reductase). The total concentration of glutathione and the ratio of reduced to oxidized glutathione defines the cytosolic redox-state and undergoes considerable changes in response to a variety of environmental stresses (16). Disulfide bridge formation in proteins frequently tracks the oxidation state of the glutathione redox buffer (16). NAB1 forms mixed disulfides with glutathione under in vitro conditions, which in turn reduces its RNA-binding activity (Fig. 1 *B* and *C*). However, future experiments have to clarify whether glutahionylation of NAB1 occurs in vivo.

NAB1 fine-tunes the translation efficiency of plastid-targeted LHCII proteins and therefore the capacity of light-harvesting and rates of photosynthesis in the chloroplast of *C. reinhardtii* cells. Under conditions where the size and composition of the LHCII complex is not properly adjusted to the prevailing external situation, the increased/decreased need for LHCII protein synthesis has to be sensed by the translation repressor NAB1 through changes in the cytosolic redox-state.

Currently the knowledge of the interplay between the plastidic redox-state, which is to a large extent determined by photosynthetic electron transport activity, and the cytosolic redox-state is limited (17). Accordingly we currently cannot depict the complete retrograde signaling pathway of NAB1 redox-regulation. However, the finding that NAB1 is regulated via reversible thiol modification, and thus the cytosolic redox environment provides important insights into the mechanisms of redox-controlled translation regulation in the cytosol of photosynthetic organisms. Redox regulation of photosynthetic gene translation in the cytosol of plant cells was reported before (18, 19), but the molecular basis and the involvement of transcript-specific RNA-binding proteins remained to be elucidated. Because the active form of NAB1 contains cysteines in the reduced thiol state, NAB1 activation is linked to reducing conditions in the cytosol, whereas its deactivation is accompanied by shifts toward the more oxidized state. Under normal, stress-free conditions the cytosol of eukaryotic cells is in a highly reduced redox state (16). A key factor, required to maintain this reduced environment, is NADPH. Major sources of NADPH supply in the cytosol of plant organisms are the glucose consuming oxidative pentose phosphate cycle (17) and NAD(P)H exporting shuttle systems in the chloroplast envelope membrane (17, 20, 21), which are reliant on photosynthetic activity in the plastid. In our current

working model, physiological conditions characterized by a sufficient provision of these photosynthates are connected to an active state of NAB1 and hence effective translation repression of LHCII transcripts. Within this model, a reduced photosynthetic performance caused by limited light supply in combination with a small antenna system oxidizes the cytosolic redox system. This in turn deactivates NAB1, thereby stimulating LHCII protein synthesis and facilitating an increase of the photosynthetic performance. For green alga, nuclear transcription activity of LHCII genes was shown to be regulated by a retrograde redox-signaling pathway emanating from the plastidic plastoquinon pool (3). With the identification of NAB1 (4) and with our recent findings, an additional mode of redox-regulated LHCII gene expression control was discovered that involves translation repression in the cytosol. Future studies targeted on the correlation between photosynthetic activity and the redox state of NAB1 could make important contributions to the understanding of retrograde signaling pathways in the context of photoacclimation processes.

Methods

Strains and Culture Conditions. Liquid cultures of *C. reinhardtii* were either cultivated mixotrophically in TAP or phototrophically in HSM medium using low-light conditions of 40 μmol m^{-2} s^{-1} continuous white-light. Cultures growing in HSM medium were bubbled with 2% CO_2. For details, see *SI Materials and Methods*.

Site-Specific Mutagenesis of *NAB1* and Transformation. Plasmid pGDNG1 was constructed by inserting the Wt *NAB1* gene into the NdeI and EcoRI cloning sites of plasmid *pGenD* (13). The plasmids pGDNG1/Cys(181/226Ser), pGDNG1/Cys(181Ser), and pGDNG1/Cys(226Ser) were generated by site-specific replacement of thymine by adenine at positions 541 and 676 of the *NAB1* gene in plasmid pGDNG1 (QuikChange Site-Directed Mutagenesis Kit; Stratagene. For primer details, see Table S1). These vectors were used to cotransform the NAB1-deficient cell line Stm3-HA-LHCBM6 (see *SI Materials and Methods*).

Coimmunoprecipitation (Co-IP) of NAB1 Targets. Co-IPs were performed with liquid TAP cultures of control strain, strain NSM1, and strain NSM2 before and after a 2-h treatment with 2 mM diamide. RT-Q-PCR was applied to quantify the amounts of coprecipitated *LHCBM6* and *ß-ACTIN* mRNA. *ß-ACTIN* served as a reference gene. For a detailed description of the procedure, see *SI Materials and Methods*.

Overexpression of Recombinant NAB1. Recombinant NAB1 was purified under native conditions according to the QIAexpressionist manual (Qiagen). Reducing conditions during the purification process were maintained by addition of 5 mM β-mercaptoethanol to binding and wash buffer. Purified protein samples were supplemented with 10 mM DTT directly after elution.

NAB1-RNA-Binding Studies. Recombinant NAB1 was subjected to oxidative treatment with 5 mM GSSG, alkylated with a 50-fold excess of NEM (N-ethylmaleimide) or a 5-fold excess of 4-vinylpyridine in respect to the sulfhydryls to blocked. Protein samples were then subjected to RNA-binding studies applying RNA probes derived from the *C. reinhardtii LHCBM6* and *psbD* genes. The probe derived from the gene *LHCBM6* was radioactively labeled, whereas the *psbD* probe was unlabeled and served as a competitor. For details, see *SI Materials and Methods*.

Subpolysome and Polysome Complex Fractionation. Polysomes were fractionated as described before (5) and RNA was extracted from all 18 sucrose gradient fractions and analyzed in an agarose-formaldehyde denaturing gel. The RNA was slot-blotted on a positively charged nylon membrane (Hybond N^+, Amersham) and hybridized with a digoxigenin-labeled *LHCBM6*- or *ß-ACTIN*-specific DNA probe. Signal intensity was quantified by densitometry and the *LHCBM6* signal of each fraction was normalized to the corresponding *ß-ACTIN* signal. For experimental details, see *SI Materials and Methods*.

Gel Electrophoresis and Immunoblotting. Proteins were separated by Tris-tricine or Tris-glycine-SDS/PAGE and detected by immunoblotting using enhanced chemiluminescence (ECL, Amersham). The NAB1-specific antiserum was obtained as already described (5) and anti-LHCII was provided by S. Jansson (Umeå, Sweden). HA-tagged proteins were detected with a HA-specific antibody (Roche Applied Science). NAB1-glutathione adducts were detected with a mouse monoclonal antibody directed against glutathione (101-A-250, Virogen). For a description of the procedure used for the detection of glutathionylated cysteines in recombinant NAB1 and the mPEG-MAL labeling procedure, see *SI Materials and Methods*.

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3.5 Chloroplast DnaJ-like proteins 3 and 4 (CDJ3/4) from *Chlamydomonas reinhardtii* contain redox-reactive Fe-S clusters and interact with stromal HSP70B. (2010)

Dorn, K.V., Willmund, F., **Schwarz, C.**, Henselmann, C., Pohl, T., Hess, B., Veyel, D., Usadel, B., Friedrich, T., Nickelsen, J. and Schroda, M. *Biochem. J.*, 427, 205 – 215

This study dealt with the role of J-domain containing proteins in the chloroplast. These proteins are thought to mediate specificity of HSP70B interactions. The identification of CDJ3, CDJ4 and CDJ5 as members of this family in the chloroplast led to the biochemical characterization of CDJ3 and CDJ4. Homologues of CDJ proteins can be found in photosynthetic eukaryotes but not in cyanobacteria. CDJ3 is a soluble protein localizing to the chloroplast and CDJ4 is found in thylakoid membranes. Levels of *cdj3/cdj4* transcripts were lowered by heat shock treatment whereas the amount of *cdj3* mRNA was increased when shifting cells from dark to the light in accordance with results of a CDJ orthologue in *Arabidopsis*. Both, CDJ3 and CDJ4 contain redox-reactive iron-sulfur clusters and interact *in vitro* with HSP70B in its ATP-bound state. CDJ3 and CDJ4 can also be detected in HSP70B-containing complexes *in vivo*. Furthermore, CDJ3 was found in stromal complexes that contain RNA, therefore reacting in size distribution to RNase treatment similar to Nac2/RBP40 complexes. Comparative analysis of CDJ3 and Nac2/RBP40 complexes in the *PRB2A* mutant – *psbD* transcrips are degraded in this mutant - showed a wild-type distribution for CDJ3. Thus, CDJ3 is interacting with HMW complexes that contain RNA but these complexes seem to lack *psbD* mRNA.

In this work, Jörg Nickelsen and I investigated distribution of RBP40 and CDJ3 in stromal extracts of *cw15* and *PRB2A* cells by gel filtration. Karolin Dorn prepared recombinant CDJ3/4 proteins and did the CDJ3/4 immunoprecipitations, dark-to-light Northern blots, cell fractionations and cross-linking experiments. Felix Willmund fractionated cells, prepared recombinant HSP70B and carried out gel filtrations with recombinant CDJ3/4. Christine Henselmann cloned the CDJ3/4 expression vectors, expressed the proteins and generated the CDJ3/4 antisera. Thomas Pohl and Thorsten Friedrich did the ESR experiments and UV–Vis spectoscopy. Barbara Hess performed the immunoprecipitations against HSP70B. Daniel Veyel performed the ATPase assays and Björn Usadel analysed the expression patterns in *Arabidopsis*. Michael Schroda carried out the heat-shock Northern blots and *in silico* analyses, prepared CDJ3/4 proteins and wrote the paper.

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online data

Chloroplast DnaJ-like proteins 3 and 4 (CDJ3/4) from *Chlamydomonas reinhardtii* contain redox-active Fe–S clusters and interact with stromal HSP70B

Karolin V. DORN*, Felix WILLMUND*, Christian SCHWARZ†, Christine HENSELMANN*, Thomas POHL‡, Barbara HESS*, Daniel VEYEL§, Björn USADEL§, Thorsten FRIEDRICH‡, Jörg NICKELSEN† and Michael SCHRODA*§[1]

*Institut für Biologie II, Biochemie der Pflanzen, Universität Freiburg, Schänzlestr. 1, Freiburg D-79104, Germany, †Molekulare Pflanzenwissenschaften, Biozentrum LMU München, Grosshaderner Strasse 2–4, Planegg-Martinsried 82152, Germany, ‡Institut für Organische Chemie und Biochemie, Albert-Ludwigs-Universität Freiburg, Albertstrasse 21, Chemiehochhaus, Freiburg D-79104, Germany, and §Max Planck Institut für Molekulare Pflanzenphysiologie, Am Mühlenberg 1, Potsdam-Golm D-14476, Germany

In the present study we report on the identification and characterization of three novel chloroplast-targeted DnaJ-like proteins CDJ3–5, which in addition to their J-domains contain bacterial-type ferredoxin domains. In sequence databases we could identify homologues of CDJ3–5 in green algae, moss and higher plants, but not in cyanobacteria. Phylogenetic analyses allowed us to distinguish two clades containing CDJ3/4 and CDJ5 that must have diverged early in the ancestor of the 'green lineage' and have further diversified later on. Molecular and biochemical analysis of CDJ3 and CDJ4 from *Chlamydomonas reinhardtii* revealed that both proteins are weakly expressed and appear to be localized to the stroma and to thylakoid membranes respectively. The low transcript levels of the *CDJ3* and *CDJ4* genes declined even further in the initial phase of heat shock, but *CDJ3* transcript levels strongly increased after a dark-to-light shift. Accordingly, the *Arabidopsis* orthologue of *CDJ5* was also found to be light-inducible and to be under strong circadian control. CDJ3 and CDJ4 proteins could both be expressed in *Escherichia coli* and had redox-active Fe–S clusters. *In vitro* cross-linking studies demonstrated that CDJ3 and CDJ4 interact with chloroplast ATP-bound HSP70B (heat-shock protein 70B), presumably as dimers, and immunoprecipitation studies showed that CDJ3/4 were also in a complex with HSP70B in *Chlamydomonas* cell extracts. Finally, CDJ3 was found in complexes with apparent molecular masses of approx. 550–2800 kDa, which appeared to contain RNA. We speculate that the CDJ3–5 proteins might represent redox switches that act by recruiting HSP70B for the reorganization of regulatory protein complexes.

Key words: bacterial ferredoxin, chloroplast chaperone, chloroplast DnaJ-like protein (CDJ), J-domain protein, redox regulation, RNA-binding protein.

INTRODUCTION

Molecular chaperones of the HSP70 (heat-shock protein 70) class are highly conserved and found in all living organisms, except for some archaea. HSP70 chaperones are involved in a plethora of different cellular functions, such as folding of newly synthesized proteins, refolding of denatured proteins after stress, protein quality control and transport of proteins across membranes, as well as assembly and disassembly of protein complexes [1]. HSP70 proteins contain two functionally interconnected domains, an N-terminal ATPase and a C-terminal substrate-binding domain. Substrate proteins typically have exposed hydrophobic regions and substrate binding to HSP70 in the ATP-bound state stimulates ATP hydrolysis [2]. This results in the tight binding of the substrate when HSP70 is in the ADP-bound state. The specificity of HSP70 function is mediated by so-called J-domain proteins, which interact with selected substrates and deliver them to HSP70 proteins in the ATP-bound state [3,4]. ATP hydrolysis is further stimulated by the interaction of the J-domain with the ATPase domain of the HSP70 partner. Exchange of ADP for ATP in most prokaryotic-type HSP70 proteins is catalysed by GrpE-type nucleotide-exchange factors [5].

Compared with the well-studied HSP70 systems in bacteria, and most compartments of the eukaryotic cell [cytosol, ER (endoplasmic reticulum) and mitochondria], rather little is known about HSP70 systems in chloroplasts. This is surprising, as chloroplasts contain thylakoid membranes, which provide the basis for almost all life on earth, and knowledge about chloroplast proteostasis is fundamental to understanding the biochemical mechanisms underlying the conversion of light energy into carbohydrates. To remedy this situation, we are studying the chloroplast HSP70 system in the unicellular green alga *Chlamydomonas reinhardtii*. *Chlamydomonas* possesses only one major chloroplast HSP70, termed HSP70B, and is therefore less complex than higher plants or mosses, which contain two and three major chloroplast HSP70 homologues respectively [6–9].

HSP70B itself is assisted by its escort protein HEP2 (HSP70 escort protein 2) to assume the functional state [10]. HSP70B co-operates with its GrpE-type nucleotide-exchange factor, CGE1 (chloroplast GrpE homologue 1) [11] and appears to be constitutively in complex with chloroplast HSP90C [12]. HSP70B has been shown to protect PSII (Photosystem II) from photo-inhibition and to play a role in the repair of photo-damaged PSII [13]. To date, two CDJ (chloroplast DnaJ-like

Abbreviations used: CDJ, chloroplast DnaJ-like protein; CGE1, chloroplast GrpE homologue 1; DTT, dithiothreitol; ECL, enhanced chemiluminescence; EST, expressed sequence tag; HEP2, Hsp70 escort protein 2; HSP, heat-shock protein; psbD, Photosystem II protein D2; PSII, Photosystem II; RBP, RNA-binding protein; ROS, reactive oxygen species; TAP, Tris/acetate/phosphate; VIPP1, vesicle-inducing protein in plastids 1.

[1] To whom correspondence should be addressed (email Schroda@mpimp-golm.mpg.de).

The nucleotide sequence data reported for CDJ3 and CDJ4 will appear in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under accession numbers GQ421467 and GQ421468 respectively.

protein) J-domain protein co-chaperones of HSP70B have been characterized. CDJ1 contains a glycine/phenylalanine-rich domain and Zn-finger substrate-binding domain, characteristic of type I J-domain proteins [14], in addition to the J-domain, and is therefore probably involved in supplying HSP70B (and HSP90C) with unfolded substrate proteins [12]. CDJ2 lacks the glycine/phenylalanine-rich and Zn-finger domains and therefore is a type III J-domain. CDJ2 binds to the VIPP1 (vesicle-inducing protein in plastids 1) and recruits the HSP70B–HSP90C chaperone complex [15,16]. HSP70B, CDJ2 and CGE1 were shown to catalyse assembly and disassembly of VIPP1 oligomeric structures and might therefore be involved in biogenesis/maintenance of thylakoid membranes [17].

In the present paper, we report on three novel chloroplast-targeted type III J-domain proteins, termed CDJ3–5, and provide a molecular and biochemical analysis of CDJ3 and CDJ4.

EXPERIMENTAL

Strains and culture conditions

C. reinhardtii strains were grown mixotrophically in TAP (Tris/acetate/phosphate) medium [18] on a rotatory shaker at 25 °C and at an illumination of ~30 μeinsteins $\cdot m^{-2} \cdot s^{-1}$ (1 einstein = 1 mole of photons). For chloroplast isolation, cells were grown in TAP medium supplemented with 0.5 % peptone.

Cloning, expression and purification of CDJ3 and CDJ4

A 623-bp DNA fragment encoding the N-terminal part of mature CDJ3 was amplified by PCR from a cDNA clone (Genbank® accession number AV628957) with primers 5′-GGACTAGTGCTCTTCTAACGCAGCTGATGAAGCAGCA-3′ and 5′-GGATTCGCGACTGCCGCCGCTGCCAGGC-3′. The PCR product was digested with SpeI and cloned into SpeI/SmaI-digested pBluescript (Stratagene), giving pMS330. pMS330 was digested with SpeI/NcoI and the resulting 501-bp fragment was ligated into a SpeI/NcoI-digested cDNA clone (Genbank® accession number AV628957), giving pMS332. Next, cDNA clone Genbank® accession number AV628957 was digested with NcoI/MfeI and the resulting 894-bp fragment was ligated into NcoI/EcoRI-digested pMS330 to generate pMS333. Finally, pMS333 was digested with SapI/XhoI and the resulting 1415-bp fragment was cloned into SapI/XhoI-digested pTYB11 (New England Biolabs), giving pMS336. The coding sequence for CDJ4 was amplified by PCR from cDNA clone Genbank® accession number AV643891 with primers 5′-GGACTAGTGCTCTTCTAACGCAAGCAGTGATGTTGCTTC-3′ and 5′-TACCACTCGAGAGCGGCGGAG-3′. The resulting 1006-bp PCR product was digested with SapI/XhoI and ligated into SapI/XhoI-digested pTYB11, generating pMS337. pMS336 and pMS337 were expressed in *Escherichia coli* ER2566 and purified by chitin-affinity chromatography according to the manufacturer's instructions (New England Biolabs). Mature CDJ3/4 were cleaved from the intein/chitin-binding domain by incubation with cleavage buffer [20 mM Tris/HCl, pH 9.0, containing 0.5 M NaCl, 1.0 mM EDTA and 50 mM DTT (dithiothreitol)] at 25 °C for 16 h and collected in five 1-ml fractions. Fractions 1–3 and 5 were pooled and subjected to three successive runs of concentration and dilution with KMH buffer (20 mM Hepes/KOH, pH 7.2, containing 80 mM KCl and 2.5 mM $MgCl_2$) using Amicon Ultra-4 tubes (Millipore). Proteins were supplemented with 10 % (v/v) glycerol and 1 mM DTT, frozen in liquid nitrogen and stored at −80 °C. Yields were approx. 300 μg of pure CDJ3/4 per litre of *E. coli* culture. Approx. 1 mg of either purified CDJ3 or CDJ4 was dissolved in 20 mM Tris/HCl, pH 8.0, containing 6 M urea and 0.5 M NaCl, and used for the generation of polyclonal rabbit antisera (Seqlab). Affinity purification of antibodies was performed as described previously [19].

Heat-shock and dark-to-light shift kinetics, RNA and protein extractions, RNA gels and hybridizations

Heat-shock and dark-to-light-shift kinetics, isolation of protein and RNA, and preparation of RNA blots were carried out as described previously [16]. Membranes were hybridized with DNA probes prepared by the random priming technique using [α-^{32}P]dCTP (Hartmann Analytic). Hybridization was performed as described previously [13]. A 2-kb NheI/AatII fragment containing the *HSP70B* coding region, the entire *CDJ3* and *CDJ4* cDNAs and a 1-kb cDNA of *CBLP* were used as probes [20]. Radioactive signals were detected with BAS-IP MS 2040 phosphorimager plates (Raytest).

SDS/PAGE and gel blot analyses

SDS/PAGE was performed as described previously [21]. For fractionation experiments, one volume of 2× Laemmli sample buffer [125 mM Tris/HCl, pH 6.8, containing 20 % (v/v) glycerol, 4 % (w/v) SDS, 10 % (v/v) 2-mercaptoethanol and 0.005 % Bromphenol Blue] was added to the samples and protein concentrations were determined with Amido Black dye [22]. Proteins in gels were stained with colloidal Coomassie (Invitrogen) or transferred on to nitrocellulose membranes (Hybond™-ECL; GE Healthcare) by semi-dry blotting using a discontinuous transfer system. Blocking and immunostaining were performed in PBS containing 3 % (w/v) non-fat dried milk and immunodetection was performed by ECL (enhanced chemiluminescence). Antisera were against HSP70B [13], CGE1 [11], mitochondrial carbonic anhydrase [23], and cytochrome *f* [24]. ECL signals were detected with Hyperfilm-ECL (GE Healthcare).

Cell fractionations

Isolation of chloroplasts and fractionation into stroma, thylakoids and low-density membranes was performed as described previously [25]. Mitochondria were isolated as described previously [26], but using a BioNebulizer™ (Glas-Col) for disrupting cells instead of vortexing with glass beads.

Immunoprecipitations

Chlamydomonas CF185 cells [13] were grown to a density of approx. 8×10^6 cells/ml and were harvested in two equal fractions by centrifugation for 5 min at 3300 ***g*** and 25 °C. The cells were either resuspended in 50 ml of pre-warmed TAP medium at 40 °C, for heat-shock, or in 50 ml of TAP medium at 25 °C, as a control. Heat-shock and control treatments were performed for 1 h under shaking, then cells were harvested and resuspended in lysis buffer [20 mM Hepes, pH 7.2, containing 10 mM KCl, 1 mM $MgCl_2$, 154 mM NaCl and 0.25× protease inhibitor cocktail (Roche)]. Cells were lysed by sonication on ice for 1 min using a 65 % duty cycle and 4.5 output control (Branson Sonifier 450). Lysates were loaded on to sucrose cushions (20 mM Hepes/KOH, pH 7.2, containing 0.6 M sucrose) and centrifuged in a TI-50 rotor for

Table 1 Properties of the CDJ3–5 proteins

The mature molecular mass was calcualated by using the transit peptide prediction according to ChloroP. aa, amino acids; –, not determined.

Name	Calculated precursor M_r (aa number)	Calculated mature M_r (aa number)	Apparent M_r in SDS/PAGE	Percentage identical/similar to mature CDJ3	Percentage identical/similar to mature CDJ4	pI mature
CDJ3	43111.3 (393)	38258.6 (353)	~34000	–	–	5.13
CDJ4	41789.1 (371)	33136.2 (306)	~38000	47 %/63%	–	4.97
CDJ5	42851.5 (383)	37737.6 (334)	–	36 %/47%	35 %/50%	5.49

30 min at 48 000 rev./min and 4 °C. Triton X-100 was added to the supernatants to a final concentration of 0.5 % and incubated for 5 min. Protein A–Sepharose beads (Sigma–Aldrich) with coupled antibodies were equilibrated in lysis buffer and incubated with the cell lysates under agitation for 1 h at 10 °C. Beads were washed four times with lysis buffer containing 0.1 % Triton X-100 and twice with 10 mM Tris/HCl, pH 7.5. Proteins were eluted by boiling for 45 s in 2× Laemmli sample buffer (for Figure 5B) or by shaking for 30 min at 25 °C with 2× Laemmli sample buffer lacking 2-mercaptoethanol (for Figure 5A).

UV–visible spectroscopy

A 200 μl aliquot of purified CDJ3 (30.5 μM) or CDJ4 (22.0 μM) in cleavage buffer was analysed in a 1-cm pathlength UV microcuvette (Brand) with a TIDAS-II diode array spectrophotometer (J&M). The samples were reduced by the addition of 5 μl (0.5 M) of sodium dithionite at 25 °C and measured instantaneously. The molar absorption coefficient at 280 nm was calculated from the amino acid sequence according to [27], giving values of 35360 $M^{-1} \cdot cm^{-1}$ for CDJ3 and 49180 $M^{-1} \cdot cm^{-1}$ for CDJ4.

Other procedures

Glutaraldehyde cross-linking was performed as described in [28] and gel filtration analysis of purified CDJ3/4 as described in [17]. Recombinant HSP70B containing a C-terminal His_6 tag was co-expressed with HEP2 in *E. coli* and purified by Ni-NTA (Ni^{2+}-nitrilotriacetate)-affinity chromatography as described previously [10]. Stromal chloroplast proteins, for size-exclusion chromatography, were extracted as described in [25]. For RNase treatment, stromal proteins were incubated for 60 min at 4 °C with 200 μg of RNase A. After application of proteins to a Superose™ 6 column (GE Healthcare), proteins were eluted with buffer (20 mM tricin/KOH, pH 7.5, containing 50 mM KCl, 5 mM ε-aminocaproic acid and 2.5 mM EDTA) using an ÄKTApurifier system (GE Healthcare) system with a flow rate of 23 cm/h. Owing to the low abundance of CDJ3, samples were concentrated using AmiconUltra centrifugal filter devices (Millipore) prior to analysis by Western blotting.

RESULTS

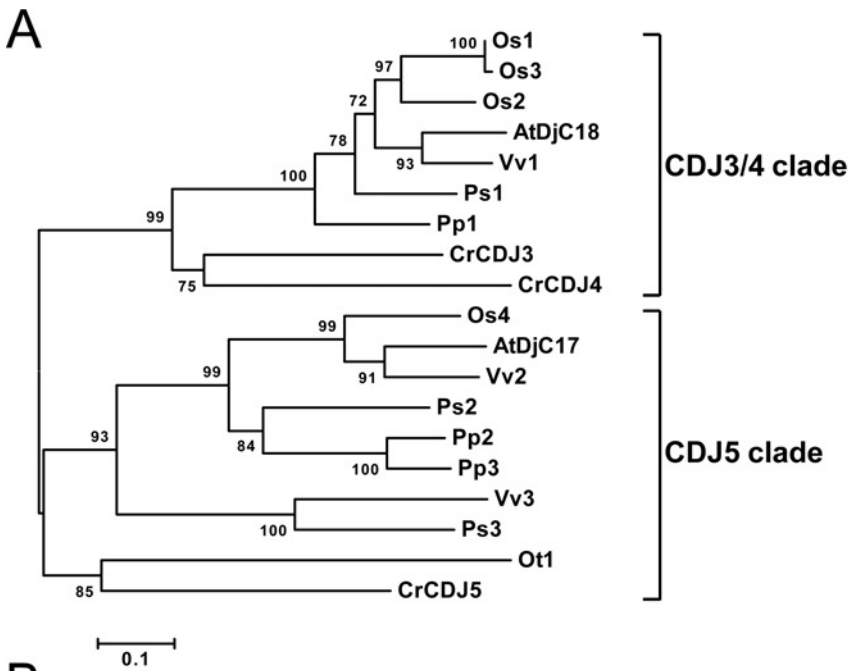
CDJ3–5 are conserved from green alga to higher plants

To find cDNAs that encode chloroplast-targeted J-domain proteins we searched *Chlamydomonas* EST (expressed sequence tag) libraries [29,30] using the amino acid sequence of the J-domain of *E. coli* DnaJ (this had led previously to the identification of CDJ1 and CDJ2, two chloroplast-targeted J-domain proteins, which interacted with chloroplast HSP70B [12,16]). We thereby identified two further ESTs that potentially encoded additional J-domain proteins with N-terminal extensions, which were predicted chloroplast transit peptides (by the ChloroP program [31]) (Figure 1B). We termed these proteins CDJ3 and CDJ4 and determined the full sequences of their cDNAs. The complete cDNA sequences revealed that the two proteins lacked the glycine/phenylalanine-rich and Zn-finger domains typical for type I J-domain proteins of the DnaJ/HSP40 prototype and therefore CDJ3 and CDJ4 were type III J-domain proteins [14]. Strikingly, both proteins contained a bacterial ferredoxin domain followed by an extended C-terminal domain of unknown function (Figure 1B). When using version 3.0 of the *Chlamydomonas* genome sequence [32] a further gene (*CDJ5*) with full EST coverage was revealed; this encodes another J-domain protein with a bacterial ferredoxin domain. Analysis by ChloroP qualifies the N-terminal sequence of CDJ5 as a potential chloroplast transit peptide. Unfortunately, as *CDJ5* was identified at a late stage of the present study it has not been characterized in detail. Information on the molecular mass of the CDJ3–5 precursors and mature proteins, and similarities between their amino acid sequences are compiled in Table 1.

Database searches revealed that true homologues of CDJ3–5 existed only in green algae, moss and higher plants, each of which encoded at least two homologues of CDJ3–5 (note that one *Ostreococcus* sequence was incomplete and therefore excluded from our analysis). It was not clear whether the diversification of the CDJ3–5 family occurred early in the development of the green lineage, or rather late, at the level of its different branches. Phylogenetic analysis of CDJ3–5 homologues from algae, moss, gymnosperms and angiosperms supported both hypotheses (Figure 1A): we could clearly distinguish two clades, one containing homologues of CDJ3 and CDJ4, the other containing homologues of CDJ5. These results suggested that the two clades must have diverged early, i.e. in the progenitor of the green lineage; however, we also found diversification within the clades, e.g. in rice, which encodes at least three members of the CDJ3/4 clade.

CDJ3 is inducible by light but not by heat stress

J-domain proteins potentially support their HSP70 chaperone partner in refolding of stress-denatured proteins. Thus it was possible that the *CDJ3/4* genes were heat-shock-inducible. As shown in Figure 2(A), the opposite was observed: both, *CDJ3* and *CDJ4* mRNA levels declined in response to heat-shock and only started to recover when the expression of heat-shock genes, e.g. *HSP70B*, was attenuated. Interestingly, *CDJ3* mRNA levels strongly increased in cells that were shifted from a 16-h dark period to low light (Figure 2B). In these conditions *CDJ4* mRNA levels were below the detection limit. Consistently, the mRNA level of the *Arabidopsis CDJ5* orthologue *atDjC17* (*AT5G23240*; extracted from publicly available microarray data) also increased after illumination (Supplementary Figure S1 at http://www.BiochemJ.org/bj/427/bj4270205add.htm). Furthermore, the mRNA levels of *atDjC17* showed strong



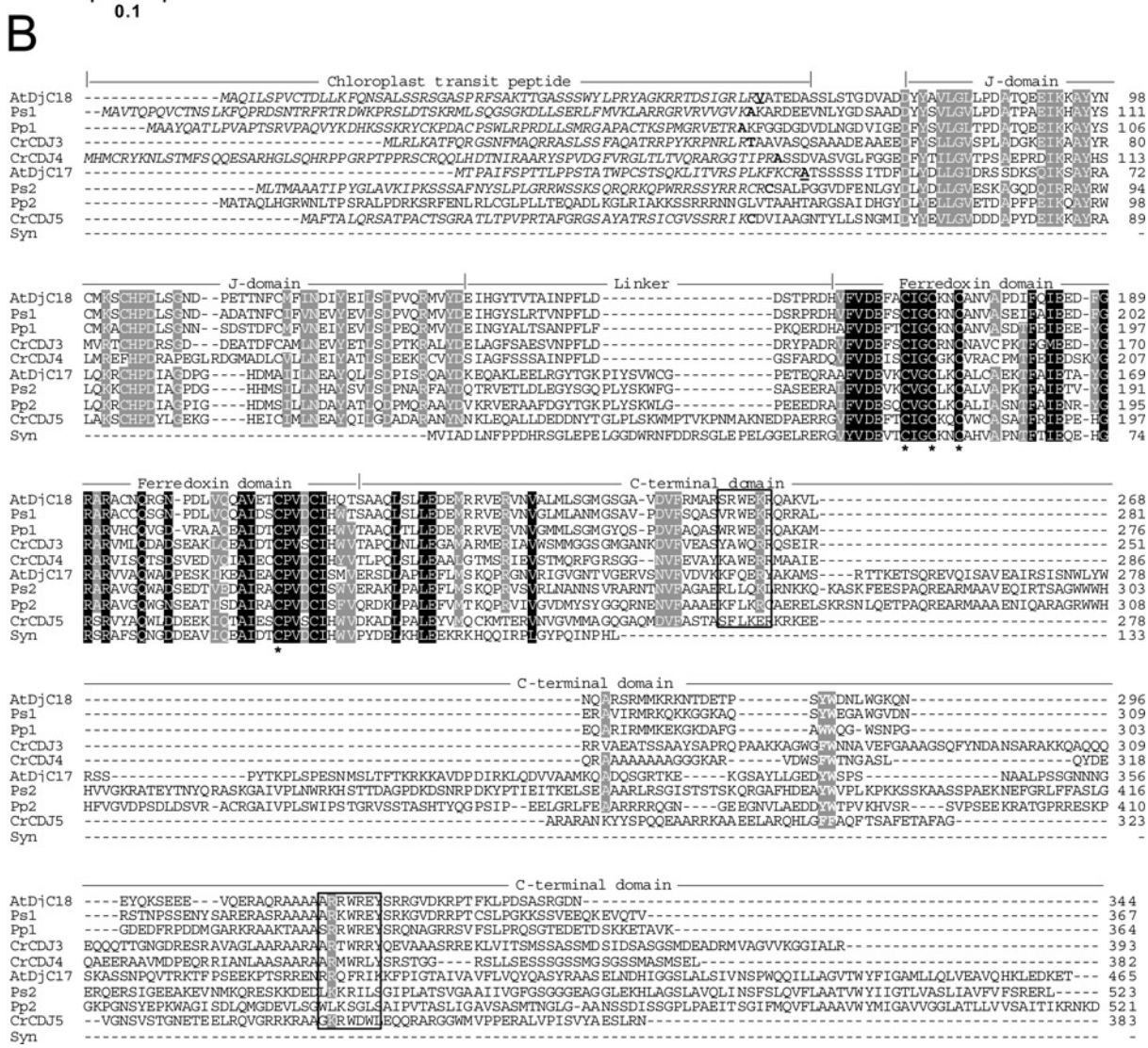


Figure 1 For legend see facing page

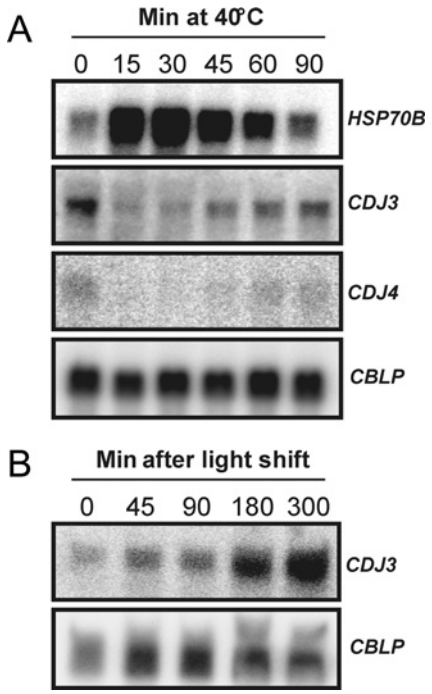


Figure 2 *CDJ3* and *CDJ4* mRNA accumulation after heat shock and dark-to-light shift

mRNA from *Chlamydomonas* wild-type cells that were exposed to a heat shock at 40 °C or that were grown for 16 h in the dark and then shifted to low-light (30 μeinsteins $\cdot m^{-2} \cdot s^{-1}$) were separated on agarose gels (10 μg per lane) and transferred on to nylon membranes. These were hybridized with probes generated from the coding regions of *HSP70B* (positive control), *CDJ3*, *CDJ4* and *CBLP* (loading control).

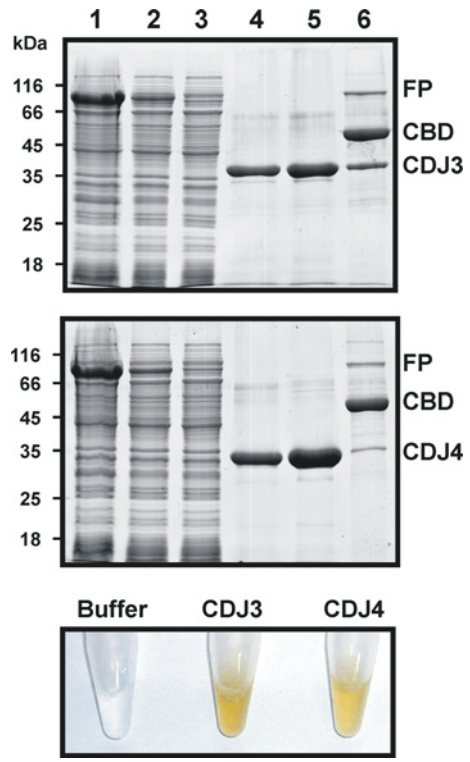


Figure 3 Heterologous expression of CDJ3 and CDJ4

CDJ3 (top panel) and CDJ4 (middle panel) were expressed as C-terminal fusions to the *S. cerevisiae* VMA intein, which contains a chitin-binding domain, purified by chitin-affinity chromatography and eluted after thiol-induced cleavage of the intein. Aliquots obtained during the purification steps were separated by SDS/PAGE (10 % gels) and stained with Coomassie Brilliant Blue. Lane 1, lysates of *E. coli* host cells after induction with IPTG (isopropyl β-D-thiogalactoside); lane 2, supernatant of cell lysates after a 20-min centrifugation at 20 000 ***g***; lane 3, flow-through; lane 4, 0.5 % of eluate (fraction 4) after thiol-induced cleavage; lane 5, 0.3 % of fractions 1–3 and 5 after concentration; lane 6, proteins remaining on the chitin column after elution. The positions of the fusion proteins (FP), cleaved CDJ3 or CDJ4 and of the *S. cerevisiae* VMA intein/chitin-binding domain (CBD) are indicated. The molecular mass in kDa is shown on the left-hand side. Pictures of eluted proteins at concentrations of 3 μg/μl (CDJ3) and 1 μg/μl (CDJ4) are shown in the bottom panel.

circadian cycling, with an approx. 30-fold increase in expression towards subjective dusk [33]. This was even more marked for *atDjC17* mRNA levels in plant rosettes grown under 12 h light/12 h dark diurnal cycles [34]. In the case of the *Arabidopsis CDJ3/4* orthologue *atDjC18* (*AT2G42750*) weaker circadian/diurnal cycling could be observed, with the expression peaking shortly after dawn. *atDjC18* was up-regulated by iron starvation [34a] and both *Arabidopsis* genes appeared to be down-regulated by sugar [34b].

CDJ3 and CDJ4 contain redox-active Fe–S clusters

To biochemically characterize CDJ3 and CDJ4 we expressed both proteins, without their predicted N-terminal chloroplast transit peptides, in *E. coli* as C-terminal fusions to the yeast VMA intein/chitin-binding domain. As shown in Figure 3, the CDJ3/4 fusion proteins were well-expressed in *E. coli*, but were barely soluble. When the fusion proteins, present in the soluble fraction, were concentrated on chitin columns, the columns became yellow-brownish, which indicated that Fe–S clusters were already assembled into the fusion proteins. Following thiol-induced cleavage, mature CDJ3 and CDJ4 proteins with apparent molecular masses of approx. 38 and 34 kDa were recovered, which corresponded well with those calculated (Table 1). The

Figure 1 Phylogenetic tree and alignment of CDJ3–5 homologues

(**A**) Phylogram based on an alignment of the amino acid sequences from CDJ3–5 and their homologues, all lacking the N-terminal extensions from their J-domains. Sequences used were from the following organisms: *Oryza sativa* (Os1–4; Genbank® accession numbers NP_001056124, NP_001044143, AAS72346 and NP_001054247 respectively), *Arabidopsis thaliana* (AtDjC17 and AtDjC18; Genbank® accession numbers NP_197715 and NP_565982 respectively), *Vitis vinifera* (Vv1–3; Genbank® accession numbers XP_002281976, CAN73797 and XP_002278893 respectively), *Picea sitchensis* (Ps1–3; Genbank® accession numbers ABK21719, ABK24669 and assembly of ESTs DR538561 and ES860441 respectively), *Physcomitrella patens* [Pp1–3; Genbank® accession number EDQ75158 (complemented with ESTs with Genbank® accession numbers FC338026 and FC448519), EDQ53967, and EDQ72847 respectively], *Ostreococcus tauri* (Ot1; Genbank® accession number CAL50030) and *Chlamydomonas reinhardtii* (CrCDJ3–5; Genbank® accession numbers GQ421467, GQ421468 and EDP07097 respectively). Phylogenetic analysis was conducted using version 4 of the MEGA program [55] on the basis of alignments made by version 1.8 of CLUSTALW. The scale bar indicates 0.1 substitutions per site. (**B**) Alignment of amino acid sequences of CDJ3–5 homologues. Sequences were limited to one representative for the CDJ3/4 and CDJ5 clades from angiosperms, gymnosperms, moss and algae from the same sources as in (**A**). The sequence of *Synechocystis sp. PCC 6803* bacterial type ferredoxin (Genbank® accession number BAA10759) is also shown. Amino acids highlighted in black are conserved in all ten proteins; those highlighted in grey are conserved in at least eight proteins. Italicized sequences represent chloroplast transit peptides as predicted by TargetP [56] or ChloroP [31] programs, with the bold underlined residue corresponding to the first amino acid of the mature protein. No prediction was obtained for Pp2. Asterisks indicate cysteine residues involved in [4Fe–4S] cluster binding [41], and boxed regions represent patches enriched in aromatic and charged residues. Alignments were determined using CLUSTALW and the GeneDoc program was used for presentation.

purified mature proteins also had a yellow-brownish colour (Figure 3).

To assay whether CDJ3 and CDJ4 contained redox-active Fe–S clusters, we characterized the UV–visible spectroscopic properties of mature CDJ3 and CDJ4. The spectra revealed broad absorption maxima at 390 nm, which bleached in the presence of sodium dithionite (Figure 4). Both the oxidized and the reduced spectra are similar to those observed for *P. aerogenes* ferredoxin [35]. The molar absorption coefficients at 390 nm of the oxidized spectra and the difference absorption coefficients at 425 nm were in the range reported for a single [4Fe–4S] cluster [36]. It was not possible to obtain an EPR signal at various temperatures and microwave power settings with either the oxidized or the reduced samples (results not shown). It is possible that the putative [4Fe–4S] clusters of CDJ3 and CDJ4 have a spin-ground state difference of $S = 1/2$. The concentration of the samples was too low to detect any contribution to the $g = 4.5$ region with the X-band spectrometer.

CDJ3 and CDJ4 interact with ATP-bound HSP70B *in vitro*

As CDJ3 and CDJ4 were predicted to be targeted to the chloroplast, it was probable that they would interact with HSP70B, which is the major (if not the only) HSP70 in the chloroplast of *Chlamydomonas* [7,8]. To test this, we performed glutaraldehyde cross-linking experiments with the purified proteins using HSP70B co-expressed with its escort protein HEP2 (and therefore functional [10]). As J-domain proteins interact with their HSP70 chaperone partners in the ATP-bound state (see e.g. [37]), we performed the cross-linking in the presence and absence of ATP. When cross-linked proteins were detected with an antiserum against HSP70B, we mainly detected HSP70B monomers (migrating slightly above the 72-kDa marker protein) and oligomers (migrating above the 170-kDa marker protein). However, we also detected cross-linked products with apparent molecular masses of approx. 140 kDa in the lanes containing ATP, HSP70B and either CDJ3 or CDJ4 (Figure 5, left-hand gel). These 140-kDa cross-linked products were also detected with antisera against CDJ3 (Figure 5, middle gel) and CDJ4 (Figure 5, right-hand gel). Hence CDJ3 and CDJ4 appear to interact with HSP70B, but only when it is in the ATP-bound state. As judged from the migration of these complexes at approx. 140 kDa they might consist of an HSP70B monomer and a CDJ3 or CDJ4 dimer. However, it is also possible that these complexes consist of monomers of HSP70B and CDJ3/4 and that the migration was retarded by cross-links that preserved higher-order structures. In addition to the 140-kDa complex with HSP70B, only monomers of CDJ3 were detected (Figure 5, middle gel). In contrast, CDJ4 existed as monomers and two oligomeric forms, which migrated at a little below and a little above the 72-kDa marker protein (Figure 5, right-hand gel). It is likely these forms represent CDJ4 dimers; in the form with higher apparent molecular mass cross-linking might have preserved higher-order structures.

We could also observe oligomer formation of CDJ4 in gel filtration experiments. As shown in Figure 6, CDJ3 and CDJ4 monomers migrated with slightly higher apparent molecular masses compared with the calculated molecular masses (approx. 47 and 42 kDa compared with approx. 38 and 33 kDa respectively). Whereas CDJ3 was only found as monomers, a small fraction of CDJ4 appeared to form oligomers. When we take into account the higher apparent molecular mass of CDJ4, the peak corresponding to a 98-kDa oligomer points to a CDJ4 dimer (Figure 6).

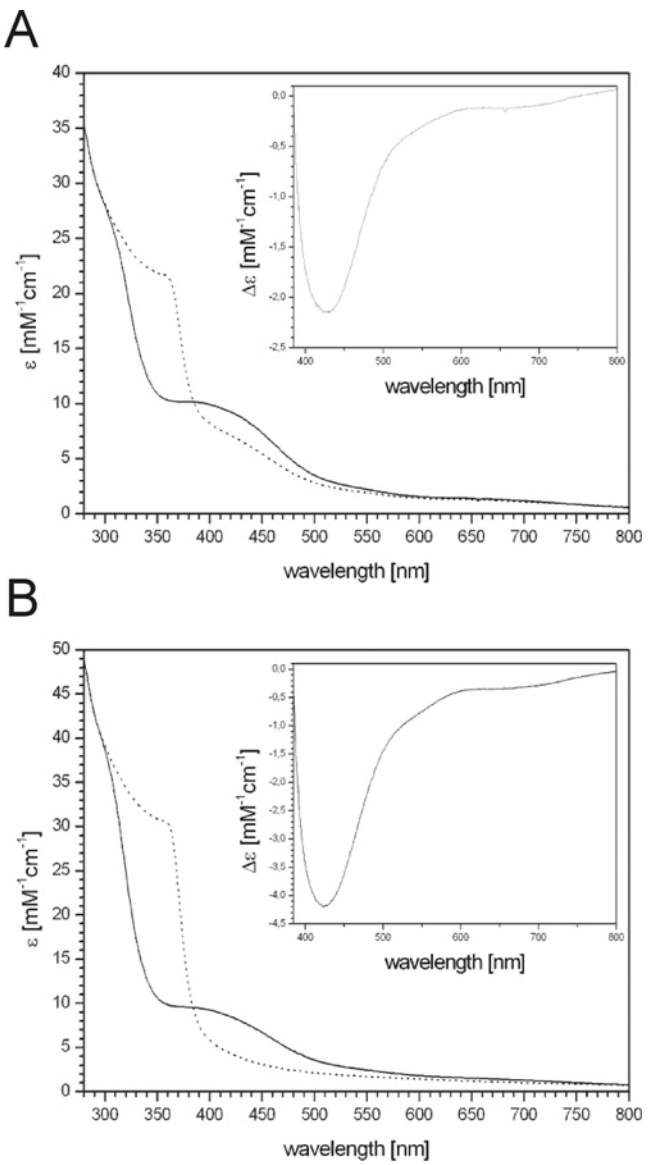


Figure 4 UV–visible spectra of purified proteins

(**A**) The spectra of oxidized (solid line) and dithionite-reduced CDJ3 (broken line) are shown. The dominant absorbance below 400 nm in the spectra of the reduced samples derives from dithionite. The inset represents the reduced-minus-oxidized difference spectrum. (**B**) Spectra of CDJ4 recorded as described in (**A**) for CDJ3.

CDJ3 and CDJ4 are very weakly expressed chloroplast proteins that locate to stroma and thylakoid membranes respectively

To test whether we could verify expression of both proteins by Western blotting we used antisera that were raised against CDJ3 and CDJ4. As shown in Figure 7, we could not, or at most barely, detect either of the two proteins in whole-cell extracts, even when we used the antisera at high concentrations. However, when we used the antisera against CDJ3 and CDJ4 to enrich the proteins by immunoprecipitation from whole-cell extracts, we could detect immunoreactive proteins with apparent molecular masses of approx. 38 and 34 kDa. These masses corresponded with those calculated for mature CDJ3 and CDJ4 respectively (Figure 7A and Table 1). In the anti-CDJ3 and anti-CDJ4 antibody immunoprecipitates we could also detect HSP70B. Hence these results suggest that the CDJ3 and CDJ4 proteins are expressed

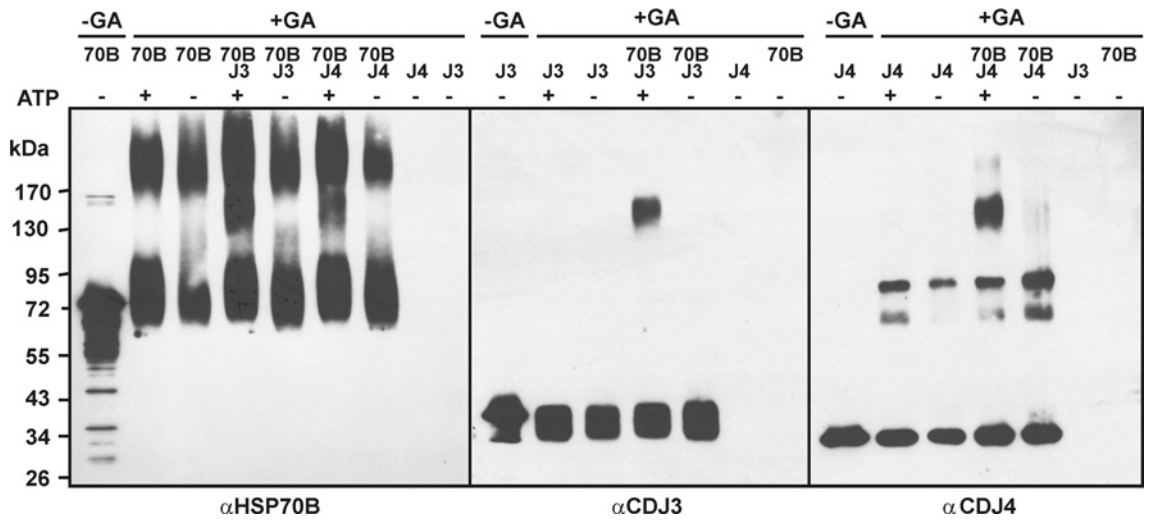


Figure 5 Glutaraldehyde-induced cross-linking of HSP70B and CDJ3/4

Purified HSP70B, CDJ3 and CDJ4 (0.5 μM) were incubated alone or in the combinations indicated for 30 min at 30 °C in the presence of 0.5 unit apyrase (ATP −) or 200 μM ATP (ATP +). Proteins were cross-linked for 15 min with 0.05 % glutaraldehyde (GA), and were separated by SDS/PAGE (4–18 % gel) and analysed by immunoblotting.

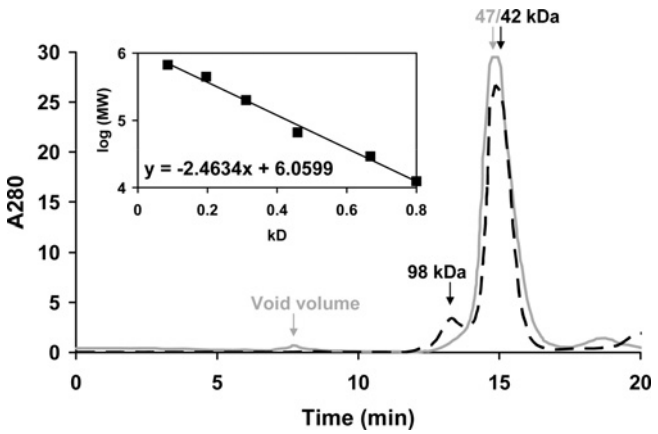


Figure 6 Gel filtration of CDJ3 and CDJ4

Purified CDJ3 (50 μg; solid grey line) and CDJ4 (50 μg; broken line) were loaded on to a Superdex 200 gel-filtration column and developed at a flow rate of 0.5 ml/min. The calibration curve using thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12.4 kDa) as marker proteins are shown in the inset. kD, k_D (partition coefficient).

at low levels in *Chlamydomonas* and that they may interact with HSP70B *in vivo*.

To verify the predicted chloroplast localization of CDJ3 and CDJ4 and to determine their suborganellar distribution, we isolated mitochondria and chloroplasts from *Chlamydomonas* cells. Chloroplasts were subsequently sub-fractioned into stroma, thylakoids and low-density membranes, which are considered to consist of inner envelopes and of transitory membranes between inner envelope and thylakoids [25]. The purity of the fractions was tested with antisera against mitochondrial carbonic anhydrase, stromal CGE1 and the integral thylakoid membrane protein cytochrome *f*. As judged from the signals obtained with these antisera (Figure 7B), chloroplasts contained mitochondrial contaminations, stroma fractions were free from thylakoid and mitochondrial contaminations, thylakoids were free from stromal contaminations, but were strongly contaminated with mitochondria, and mitochondria were pure. CDJ3 was detected in the chloroplast and stromal fractions, whereas CDJ4 was weakly detected in chloroplast and strongly detected in thylakoid fractions (Figure 7B).

CDJ3 appears to be in complex with chloroplast transcripts

As outlined in more detail in the Discussion section, one possible function of CDJ3–5 might be the redox-regulation of transcription/translation initiation and/or transcript stability. If this was the case, we would expect CDJ3–5 to be associated with chloroplast RNA transcripts. To test this hypothesis, we subjected untreated stromal extracts and extracts that have been treated with RNase to a gel-filtration assay and monitored the fractions for the presence of CDJ3. We limited our analysis to CDJ3 given we could detect it in the stroma. We also monitored gel-filtration fractions for the presence of RBP40 (RNA-binding protein 40) as a control {RBP40 specifically binds to the *psbD* (PSII protein D2) mRNA; [38]}. As expected, in untreated stroma RBP40 was detected in fractions with complexes ranging from approx. 160 to 2800 kDa, whereas in RNase-treated stroma it shifted into fractions with complexes in the approx. 160–550 kDa range (Figure 8). Interestingly, CDJ3 in untreated stroma was detected in fractions with complexes ranging from approx. 550 to 2800 kDa. RNase treatment extended the fractions in which CDJ3 was detected down to the 160-kDa region, at the expense of material in the higher-molecular-mass range. This suggests that some of the complexes containing CDJ3 are associated with RNA.

To test whether CDJ3 was binding to *psbD* transcripts, e.g. in association with RBP40, we isolated stromal fractions from the *PRB2A* mutant. In this mutant, the 7-bp PRB2 sequence in the 5′ UTR (untranslated region) of the *psbD* mRNA is mutated, leading to a specific destabilization of the *psbD* transcript [39,40]. As shown in Figure 8, RBP40 in the *PRB2A* mutant was detected only in stroma fractions containing complexes in the approx. 160 to 660 kDa range, corroborating the specific interaction of RBP40 with the *psbD* message [38]. As the distribution of CDJ3 was not altered in stroma fractions from the *PRB2A* mutant, CDJ3 appears to be associated with transcripts other than *psbD*.

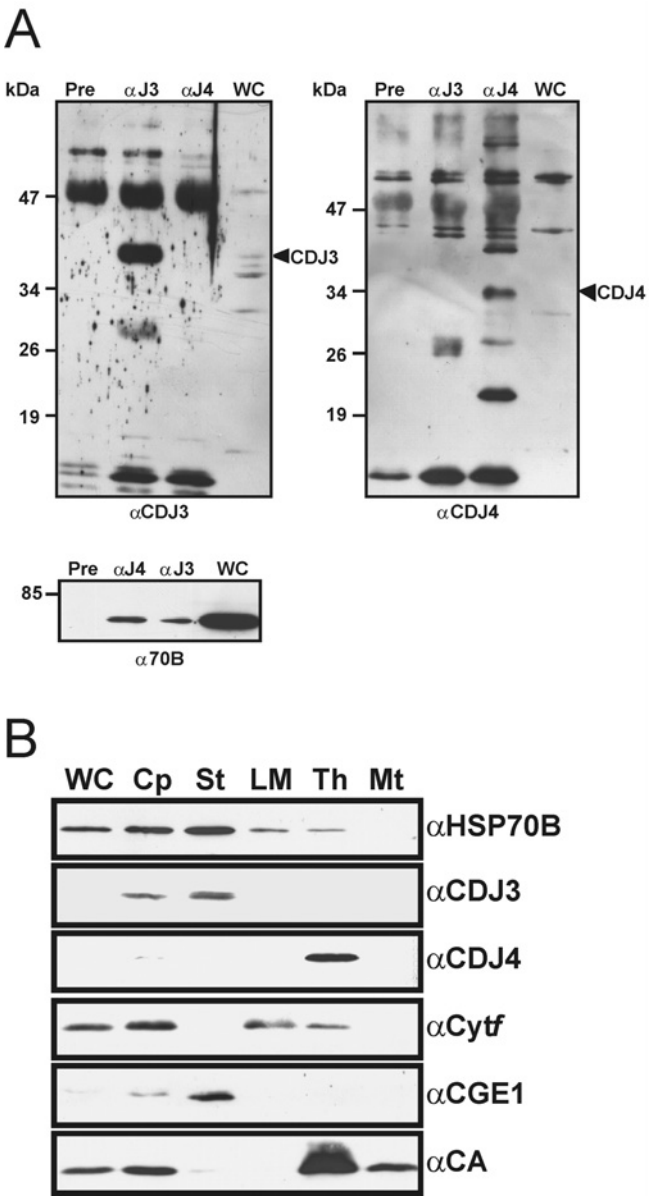


Figure 7 Enrichment and subcellular localization of CDJ3/4

(**A**) Enrichment of CDJ3 and CDJ4 from whole-cell extracts. *Chlamydomonas* whole cells were lysed by sonication in the presence of 2 % (v/v) Triton X-100 and solubilized material was separated from non-soluble matter by centrifugation through a 0.6 M sucrose cushion. The supernatant was incubated with Sepharose beads coupled to pre-immune serum (Pre) or to anti-CDJ3 (αJ3) and anti-CDJ4 antisera (αJ4). Whole-cell proteins (WC) and precipitated proteins were separated by SDS/PAGE (7.5–15 % gels) and analysed by immunoblotting with the indicated antibody. (**B**) Subcellular localization of CDJ3/4. *Chlamydomonas* chloroplasts (Cp) were separated into soluble stroma (St), low-density membranes (LM) and thylakoid membrane (Th) fractions. Mitochondria (Mt) were isolated from the same strain. Proteins (7 μg) from whole cells (WC) and subfractions were separated by SDS/PAGE (7.5–15 % gels) and analysed by immunoblotting with antisera against HSP70B, CDJ3, CDJ4, thylakoidal cytochrome *f* (αCytf), stromal CGE1 and mitochondrial carbonic anhydrase (αCA). Antibodies against CDJ3 and CDJ4 were affinity-purified.

DISCUSSION

In the present paper we report on the molecular and biochemical analysis of CDJ3 and CDJ4, two novel J-domain proteins encoded by the *Chlamydomonas* genome. We present several lines of evidence to show that CDJ3/4 are chloroplast-targeted co-chaperones of chloroplast HSP70B. First, CDJ3/4, like all their homologues in other algae, moss and higher plants, contained N-terminal extensions that the ChloroP/TargetP programs predicted to be chloroplast transit peptides. The apparent molecular masses of mature CDJ3/4 observed by SDS/PAGE (Figure 7A) corresponded with those calculated for the processed proteins (Table 1). Accordingly, fractionation experiments revealed that mature CDJ3 and CDJ4 are targeted to the chloroplast, where they are localized to stroma and thylakoids respectively (Figure 7B), with chloroplast HSP70B [11,12,15,16,19]. Secondly, HSP70B co-immunoprecipitated with both CDJ3 and CDJ4 from *Chlamydomonas* cell extracts (Figure 7A), and recombinant HSP70B was found to interact with recombinant CDJ3 and CDJ4 in the ATP-bound state *in vitro* (Figure 5). Thirdly, recombinant CDJ3/4 stimulated the ATPase activity of HSP70B, albeit at a weaker extent than the *bona fide* chloroplast DnaJ homologue CDJ1 (results not shown).

Evolution of ferredoxin-containing J-domain co-chaperones

A striking feature of CDJ3 and CDJ4 is that they contain redox-active Fe–S clusters, which according to their spectral properties and amino acid sequences appear to be of the bacterial ferredoxin [4Fe–4S] type (Figures 1, 3 and 4; [35,36,41]). The presence of a ferredoxin domain within a J-domain co-chaperone is highly unusual. Accordingly, we have found genes encoding homologues of CDJ3/4 only in algae, moss and higher plants (Figure 1). We also found *Chlamydomonas* harbours a third member of this gene family, which we termed *CDJ5* and whose deduced amino acid sequence is more distantly related to those of CDJ3 and CDJ4 (Table 1). Each plant species analysed encodes at least two members of the CDJ3–5 family, of which at least one belongs to the CDJ3/4 or CDJ5 subfamilies (Figure 1).

Interestingly, proteins containing both a J-domain and a bacterial ferredoxin domain are also encoded by the genomes of several members of the mesophilic *Crenarchaeota* (now also referred to as *Thaumarchaeota*) [42–44]. However, the domain architecture of the archaeal proteins differs from that of the CDJ3–5 homologues; J- and ferredoxin domains are separated by a linker of 22–41 amino acids in the CDJ3–5 homologues, but this linker may contain up to 117 amino acids in the archaeal proteins (Figure 1B and Supplementary Figure S2 at http://www.BiochemJ.org/bj/427/bj4270205add.htm). Instead of this extended linker, the CDJ3–5 homologues contain C-terminal domains of up to approx. 300 amino acids, which are absent in the archaeal proteins. Within their linkers, the archaeal proteins contain conserved patches of aromatic amino acids flanked by positively and negatively charged residues that, at a lower level of conservation, are also found in the C-terminal domains of CDJ3–5. This might indicate that functions exerted by the archaeal linker are located in the C-terminal domains of CDJ3–5.

Genes encoding DnaK and its co-chaperones appear to be absent from the genomes of hyperthermophilic *Crenarchaeota* and have probably been transferred horizontally from bacteria to mesophilic euryarchaeota; this might have been a prerequisite for adaptation to life at lower temperatures [44]. Regarding the evolution of the ferredoxin-containing J-domain co-chaperones, three scenarios are possible: (i) that the ancestor of CDJ3–5 appeared early during the evolution of the green lineage and was then acquired by a crenarchaeote by horizontal gene transfer; (ii) that the J-domain–ferredoxin fusion occurred first in crenarchaeotes after they had acquired components of the bacterial DnaK system and was then transferred to an early ancestor of the green lineage, giving rise to CDJ3–5; or (iii) that the J-domain–ferredoxin fusion occurred independently in crenarchaeotes and an early ancestor of the green lineage.

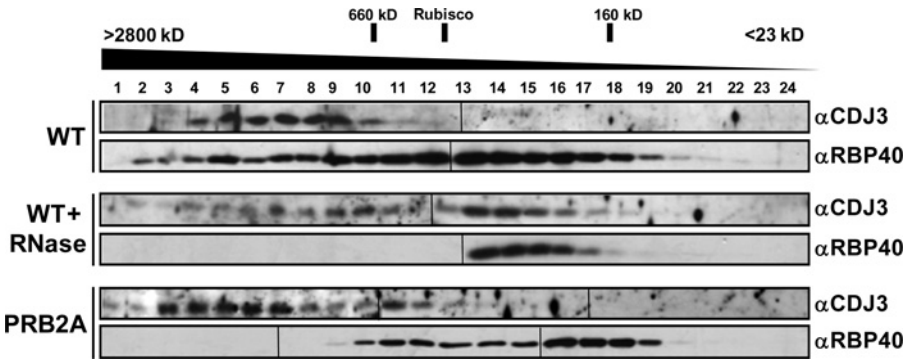


Figure 8 Gel-filtration analysis of CDJ3 complexes

Stromal proteins were separated according to their native size by size-exclusion chromatography before being subjected to SDS/PAGE followed by Western blot analysis using the indicated antisera. Analysed protein extracts included wild-type stromal proteins without (WT) and with RNase (WT + RNase) treatment and those from the *PRB2A* mutant lacking the *psbD* mRNA (PRB2A). Elution profiles of marker proteins (in kDa) are given at the top, together with the respective fraction numbers.

Phylogenetic analyses suggest that the first scenario is the most probable (D. Moreira and P. Lopez-Garcia, personal communication). In any case, development/maintenance of these specialized co-chaperones is likely to be the solution to a selective pressure similarly affecting mesophilic crenarchaeotes and members of the green lineage.

In fact, an HSP70 homologue (HscA), a J-domain protein (HscB) and a ferredoxin (Fdx) are encoded in close proximity on the bacterial *isc* operon and they themselves, and their mitochondrial homologues Ssq1, Jac1 and Yah1 respectively, are essential for Fe–S cluster biogenesis [45–47]. The HscB/Jac1 J-domain proteins facilitate the interaction of HscA/Ssq1 with scaffold proteins that bind Fe–S cluster intermediates. The chaperones may assist cluster formation by maintaining the scaffold proteins in a conformation suitable for cluster assembly or, alternatively, they may facilitate the transfer of the cluster from the scaffold to an acceptor apoprotein [45]. The yeast mitochondrial ferredoxin homologue Yah1 was suggested to provide the reducing power for an essential step in cluster biogenesis, e.g. for cysteine reduction, iron reduction or reduction of cluster intermediates prior to release from the scaffold proteins [47]. If the chaperones were indeed involved in the release of cluster intermediates and ferredoxin was essential for prior reduction, the integration of the J-domain co-chaperone and ferredoxin into the same polypeptide might have made these processes more efficient.

Possible functions of CDJ3–5

A role for the stromal HSP70 chaperone system in Fe–S cluster biogenesis might be supported by the finding that stromal HSP70s are essential [9], which is a typical feature shared by all proteins with important roles in Fe–S cluster biogenesis [48]. However, there are arguments against this hypothesis. First, the Fdx/Yah1 proteins, for which homologues in *Arabidopsis* and *Chlamydomonas* mitochondria exist [49], contain [2Fe–2S] clusters with a polypeptide fold distinct from that of [4Fe–4S]-type clusters [50]. Secondly, if CDJ3–5 were involved in such a fundamental and conserved process as Fe–S cluster biogenesis, it would be surprising to find so many family members only in the green lineage and not in cyanobacteria or non-green algae. Thirdly, a possible role for CDJ3 in Fe–S cluster biogenesis is difficult to explain in light of its observed association with RBP complexes (Figure 8).

We can envision functions for CDJ3–5 other than in Fe–S cluster biogenesis. We found *CDJ3* to be strongly induced by light and the *Arabidopsis* orthologue of *CDJ5* (*atDjC17*) was light-inducible and under strong circadian control (Figure 2 and Supplementary Figure S1). We have also shown that the Fe–S clusters in CDJ3/4 are redox-active (Figure 4), both proteins interact with HSP70B (Figures 5 and 7A) and CDJ3 appears to be part of an RBP complex (Figure 8). J-domain co-chaperones interact via specialized domains with specific substrate proteins and deliver them to their HSP70 partner for processing [3,4]. Hence, it is tempting to speculate that CDJ3–5 interact with specific substrates via their C-terminal domains and that substrate binding or delivery to HSP70B occurs only at a redox state defined by their ferredoxin domains, e.g. via an internal conformational change or a reduction step. The light inducibility of CDJ3 might suggest that redox signals inflicted by light, such the oxidation state of the thioredoxin system or the accumulation of ROS (reactive oxygen species), determine the oxidation state of CDJ3–5 and, therefore, whether substrate processing by HSP70B occurs. The association of CDJ3 with RNA again might point to a chaperone-mediated remodelling of RBP complexes that might be involved in translation initiation/elongation or mRNA stability. These complexes are also found in the stroma and are associated with thylakoids [51], where CDJ3/4 and HSP70B are also located (Figure 7B).

Well-studied examples of chaperone-mediated remodelling of replication initiation complexes are known from *E. coli*, where DnaK and DnaJ monomerize RepA dimers and dissociate DnaB helicase–λP complexes to trigger replication of plasmid P1 and λ phage respectively [52,53]. By means of their Fe–S clusters, the transcription and translation regulators SoxR, FNR, aconitase and IscR sense signals, such as ROS, NO, cellular oxygen or iron concentrations, and relay them to transcriptional or translational activities [54]. In fact, gene expression in the chloroplast is mainly regulated by nuclear-encoded factors at the level of translation initiation and/or transcript stability and, therefore, is distinct from that in cyanobacteria [51]. As this post-transcriptional regulation of the expression of many chloroplast genes, including *psbA* (PSII protein D1), *psbD* and *rbcL* (ribulose bisphosphate carboxylase, large subunit), is strongly regulated by light [51], we propose that CDJ3–5 might represent such nuclear-encoded factors that act as redox switches by recruiting HSP70B for the reorganization of regulatory protein complexes.

AUTHOR CONTRIBUTION

Karolin Dorn prepared CDJ3/4 proteins and performed the immunoprecipitations, dark-to-light-shift Northern blots, cell fractionations and cross-linking experiments. Felix Willmund prepared HSP70B, ran gel filtrations with CDJ3/4 and performed cell fractionations. Christian Schwarz and Jörg Nickelsen performed and interpreted the gel-filtration experiments with stromal extracts. Christine Henselmann cloned the expression vectors and generated the CDJ3/4 antisera. Thomas Pohl and Thorsten Friedrich performed and interpreted the UV–visible spectoscopy and ESR measurements. Barbara Hess performed the immunoprecipitations. Daniel Veyel performed the ATPase assays. Björn Usadel analysed the expression patterns in *Arabidopsis*. Michael Schroda co-ordinated the work, ran the heat-shock Northern blots, performed phylogenetic analyses and alignments, prepared CDJ3/4 proteins and wrote the paper.

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online data

SUPPLEMENTARY ONLINE DATA

Chloroplast DnaJ-like proteins 3 and 4 (CDJ3/4) from *Chlamydomonas reinhardtii* contain redox-active Fe–S clusters and interact with stromal HSP70B

Karolin V. DORN*, Felix WILLMUND*, Christian SCHWARZ†, Christine HENSELMANN*, Thomas POHL‡, Barbara HESS*, Daniel VEYEL§, Björn USADEL§, Thorsten FRIEDRICH‡, Jörg NICKELSEN† and Michael SCHRODA*§[1]

*Institut für Biologie II, Biochemie der Pflanzen, Universität Freiburg, Schänzlestr. 1, Freiburg D-79104, Germany, †Molekulare Pflanzenwissenschaften, Biozentrum LMU München, Grosshaderner Strasse 2–4, Planegg-Martinsried 82152, Germany, ‡Institut für Organische Chemie und Biochemie, Albert-Ludwigs-Universität Freiburg, Albertstrasse 21, Chemiehochhaus, Freiburg D-79104, Germany, and §Max Planck Institut für Molekulare Pflanzenphysiologie, Am Mühlenberg 1, Potsdam-Golm D-14476, Germany

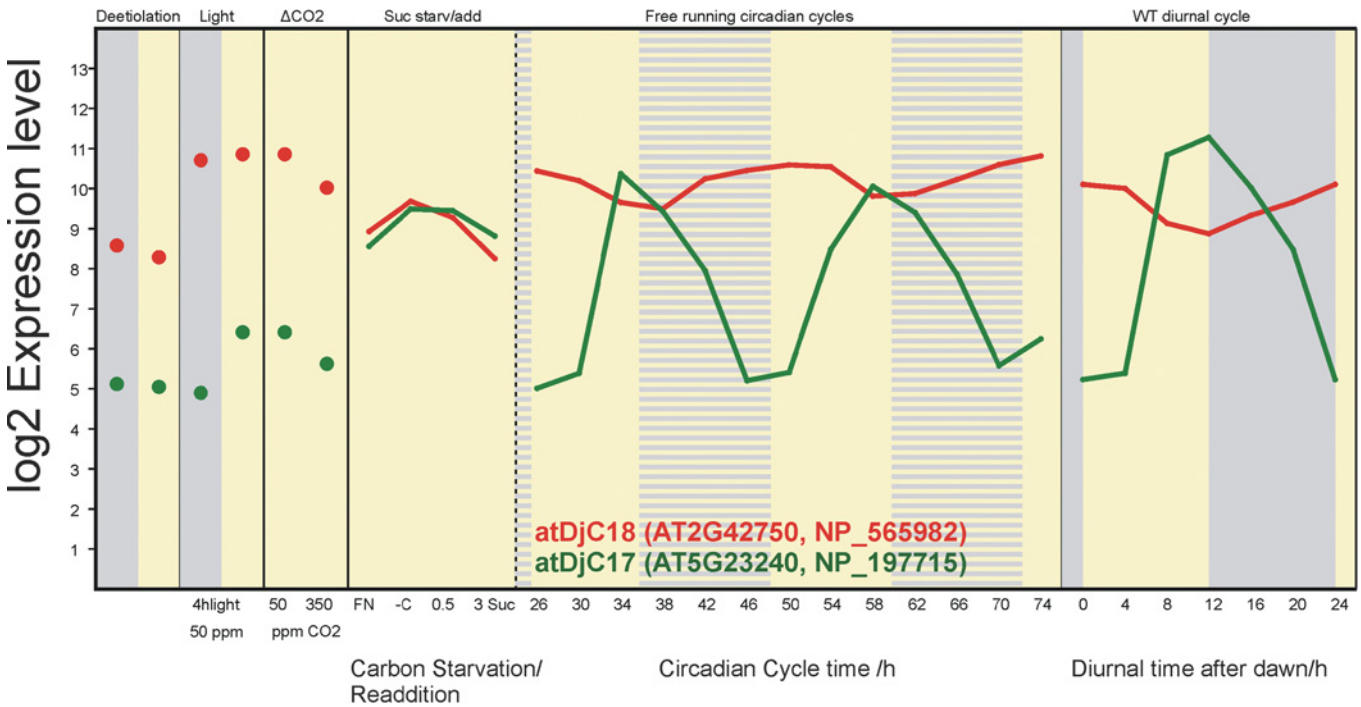

Figure S1 Circadian regulation of *Arabidopsis CDJ3–5* homologues

The scaled mRNA expression levels of *AtDjC17* and *AtDjC18* in various microarrays pertaining to sugar, light and cycles are shown. The Figure shows (from left to right): the response of seedlings to light from the Atgenexpress data set; plants left in the dark compared with the expression after plants were shifted from the dark to the light at compensation point CO_2; plants shifted from the dark to the light at compensation point or ambient CO_2; the expression of *AtDjC17* and *AtDjC18* in a seedling culture grown under full nutrition (FN), carbon starvation (-C), and 30 min or 3 h of sucrose re-addition; the expression in seedlings in constant light after entraining a 12 h light/12 h dark cycle where subjective night phases are indicated by striped grey lines; and the expression throughout the diurnal cycle, where the light and dark phases are indicated by yellow and grey backgrounds respectively.

[1] To whom correspondence should be addressed (email Schroda@mpimp-golm.mpg.de.)

The nucleotide sequence data reported for CDJ3 and CDJ4 will appear in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under accession numbers GQ421467 and GQ421468 respectively.

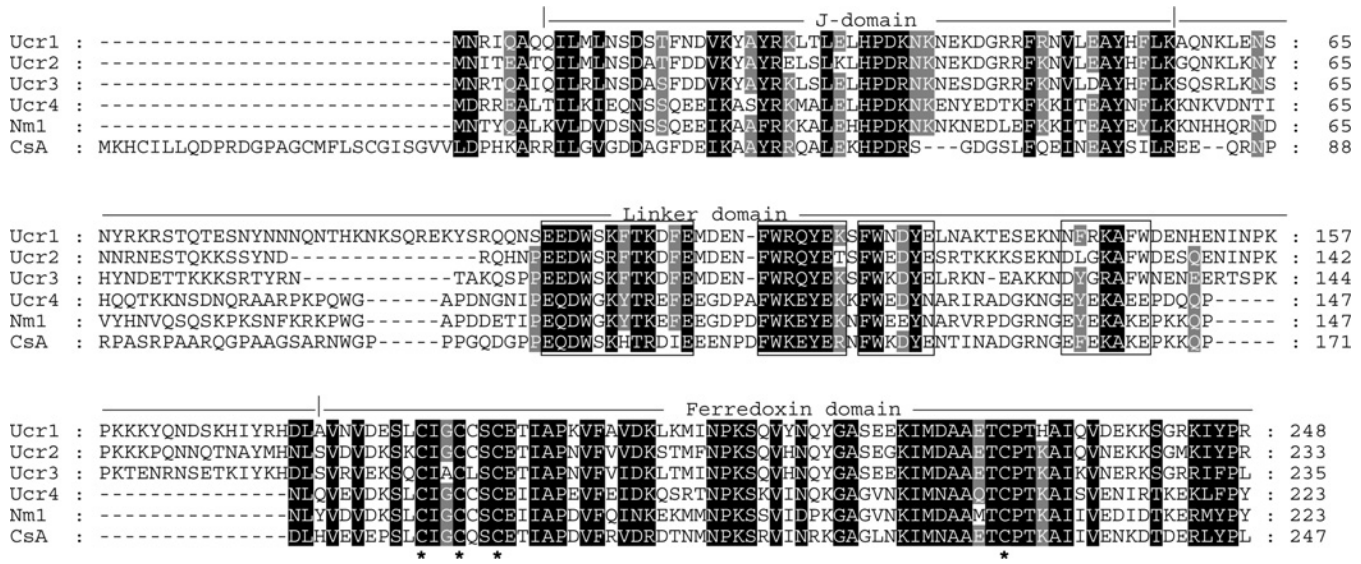

Figure S2 Alignment of J-domain/bacterial ferredoxin domain proteins from mesophilic crenarchaeota (Thaumarchaeota)

The sequences used were from the following organisms: uncultured crenarchaeote DeepAnt-EC39 (Ucr1; NCBI accession number AAR24498); uncultured marine crenarchaeote AD1000-207-H3 (Ucr2; *Genbank*® accession number ACF09820); uncultured marine crenarchaeote AD1000-56-E4 (Ucr3; NCBI accession number ACF09658); uncultured crenarchaeote 74A4 (Ucr4; *Genbank*® accession number AAK96090); *Nitrosopumilus maritimus* SCM1 (Nm1; NCBI accession number YP_001582358); and *Cenarchaeum symbiosum* A, (CsA; NCBI accession number YP_875357). Amino acids highlighted in black are conserved in all six proteins; those highlighted in grey are conserved in at least five of them. Asterisks indicate cysteine residues involved in [4Fe–4S] cluster binding and boxed regions represent patches enriched in aromatic and charged residues.

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3.6 MRL1, a conserved pentatricopeptide repeat protein, is required for stabilization of *rbcL* mRNA in *Chlamydomonas* and *Arabidopsis*. (2010)

Johnson, X., Wostrikoff, K., Finazzi, G., Kuras, R., **Schwarz, C.**, Bujaldon, S., Nickelsen, J., Stern, D.B., Wollman, F.A. and Vallon, O. *Plant Cell*, 22, 234 – 248

In this publication, the *Chlamydomonas* nuclear mutant *mrl1* was investigated which fails to accumulate *rbcL* mRNA despite unaffected synthesis rates of this transcript. The corresponding gene carrying the mutation was found to encode a conserved PPR protein named MRL1. MRL1 was found in HMW complexes of stromal proteins isolated under native conditions. Due to the bound *rbcL* mRNA its size distribution changed in the presence of RNase and in a *ΔrbcL* mutant. By using chimeric reporter gene constructs, it was confirmed that the 5`region of *rbcL* transcripts is the target of MRL1. *Arabidopsis* mutants lacking the orthologue MRL1 with the same RNA target completely retained their primary transcripts but were unable to process or stabilize them. Thus, our data suggest that MRL1 protects secondary *rbcL* transcripts against degradation.

I contributed to this publication by performing the biochemical analyses in *C. reinhardtii*. Xenie Johnson generated the *C. reinhardtii mrl1* mutants, did the algal transformations as well as the molecular biological experiments. For biophysical analyses, she worked together with Giovanni Finazzi. Katia Wostrikoff did the *Arabidopsis* experiments and provided the paAFFF vector. Richard Kuras also participated in mutant construction, whereas Sandrine Bujaldon constructed the *ΔrbcL* strain. Olivier Vallon performed the genetic crosses and the computational analyses. The manuscript was written by Xenie Johnson, Katia Wostrikoff, Giovanni Finazzi and Olivier Vallon with advice of Richard Kuras, Jörg Nickelsen, David Stern and Francis-André Wollman under Olivier Vallon’s supervision.

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MRL1, a Conserved Pentatricopeptide Repeat Protein, Is Required for Stabilization of *rbcL* mRNA in *Chlamydomonas* and *Arabidopsis* [C][W]

Xenie Johnson,[a] Katia Wostrikoff,[b,1] Giovanni Finazzi,[a,2] Richard Kuras,[a] Christian Schwarz,[c] Sandrine Bujaldon,[a] Joerg Nickelsen,[c] David B. Stern,[b] Francis-André Wollman,[a] and Olivier Vallon[a,3]

[a] Centre National de la Recherche Scientifique, Unité Mixte de Recherche 7141/Université Pierre et Marie Curie, Institut de Biologie Physico-Chimique, Paris 75005, France

[b] Boyce Thompson Institute for Plant Research, Cornell University, Ithaca, New York 14853

[c] Biozentrum Ludwig-Maximilian Universität München, D-82152 Planegg-Martinsried, Germany

We identify and functionally characterize MRL1, a conserved nuclear-encoded regulator of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. The nonphotosynthetic *mrl1* mutant of *Chlamydomonas reinhardtii* lacks ribulose-1,5-bisphosphate carboxylase/oxygenase, and the resulting block in electron transfer is partially compensated by redirecting electrons toward molecular oxygen via the Mehler reaction. This allows continued electron flow and constitutive nonphotochemical quenching, enhancing cell survival during illumination in spite of photosystem II and photosystem I photoinhibition. The *mrl1* mutant transcribes *rbcL* normally, but the mRNA is unstable. The molecular target of MRL1 is the 5′ untranslated region of *rbcL*. MRL1 is located in the chloroplast stroma, in a high molecular mass complex. Treatment with RNase or deletion of the *rbcL* gene induces a shift of the complex toward lower molecular mass fractions. MRL1 is well conserved throughout the green lineage, much more so than the 10 other pentatricopeptide repeat proteins found in *Chlamydomonas*. Depending upon the organism, MRL1 contains 11 to 14 pentatricopeptide repeats followed by a novel MRL1-C domain. In *Arabidopsis thaliana*, MRL1 also acts on *rbcL* and is necessary for the production/stabilization of the processed transcript, presumably because it acts as a barrier to 5′>3′ degradation. The *Arabidopsis mrl1* mutant retains normal levels of the primary transcript and full photosynthetic capacity.

INTRODUCTION

The biogenesis of the genome-containing organelles, chloroplasts and mitochondria, is governed by protein factors encoded in the nucleus, most of which probably remain to be unraveled (Barkan and Goldschmidt-Clermont, 2000). These factors control chloroplast gene expression at the posttranscriptional, translational, and posttranslational levels, leading to the concerted production of nuclear- and chloroplast-encoded subunits of the photosynthetic enzymes. Each chloroplast gene appears to be controlled by a suite of nucleus-encoded factors, usually specific to a single or a few genes. In some cases, these factors have been shown to accumulate in limiting amounts for the production of their target protein (Raynaud et al., 2007). Many regulators of organelle gene expression interacting with mRNA belong to families of repeat-containing proteins. Among them are the RNA binding tetratricopeptide repeat proteins, such as NAC2 and HCF107 (Boudreau et al., 2000; Sane et al., 2005); however, the majority of sequence-specific RNA-interacting regulators described in chloroplasts and mitochondria belong to another family of repeat proteins, the pentatricopeptide repeat (PPR) family.

PPR proteins are characterized by the presence of repeated degenerated units of 35–amino acid residues. Based on similarity to the α-solenoid superfamily, it is believed that each PPR folds into a pair of antiparallel α-helices, whose stacking forms a superhelical structure able to bind in its groove an extended RNA molecule (Delannoy et al., 2007). To date, PPR proteins have been found in all eukaryotes, but the family is particularly expanded in land plants (for a recent review, see O'Toole et al., 2008), with >450 in *Arabidopsis thaliana*, located either in chloroplasts or in mitochondria (Lurin et al., 2004). PPR proteins function in RNA processing, intron splicing, RNA editing, and translation. Some, like the E-class PPR proteins, are composed of a string of PPR motifs and a characteristic C-terminal domain, which may recruit, by protein–protein interaction, an effector to the correct site (for a recent review, see Schmitz-Linneweber and Small, 2008). Others, like CRP1 and PGR3, have no recognizable

[1] Current address: Centre National de la Recherche Scientifique, Unité Mixte de Recherche 7141/UPMC, Institut de Biologie Physico-Chimique, 13 Rue Pierre et Marie Curie, Paris 75005, France.

[2] Current address: Centre National de la Recherche Scientifique, Unité Mixte de Recherche 5168, Commissariat à l'Energie Atomique, 17 Rue des Martyrs, 38054 Grenoble, France.

[3] Address correspondence to ovallon@ibpc.fr.

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[C] Some figures in this article are displayed in color online but in black and white in the print edition.

[W] Online version contains Web-only data.

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domains apart from the PPR motifs. These two proteins have been implicated both in RNA stability and in translation (Yamazaki et al., 2004; Schmitz-Linneweber et al., 2005), similarly to MCA1, which interacts with *petA* and was the first PPR protein characterized in *Chlamydomonas reinhardtii* (Loiselay et al., 2008).

The PPR code, which would link the succession and amino acid sequence of the repeats with the nucleotide sequence of the target mRNA, remains to be established. Immunoprecipitation and in vitro binding studies have delineated binding sites for maize (*Zea mays*) CRP1, PPR5, and PPR10 (Schmitz-Linneweber and Small, 2008; Williams-Carrier et al., 2008; Pfalz et al., 2009). The general model that emerges for the stabilization mechanism is that binding of the PPR protein shelters a specific region of RNA from nucleases, in the manner of a protein cap. At the same time, the protein may facilitate other processes, such as splicing in the case of PPR5.

In this study, we describe MRL1, a PPR protein found in plants and green algae, that controls the accumulation of the *rbcL* mRNA at a posttranscriptional stage and, hence, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) biogenesis. The contrasting phenotypes of the *mrl1* mutants of *Arabidopsis* and *Chlamydomonas* underline the high metabolic flexibility of the latter unicellular organism that developed photoprotection strategies in the absence of CO_2 fixation by Rubisco.

RESULTS

The *Chlamydomonas mrl1* Mutant Fails to Accumulate *rbcL* mRNA

The *Chlamydomonas mrl1* mutant was isolated from a collection of paromomycin-resistant transformants obtained by random insertion of the *aphVIII* gene, followed by screening for a non-phototrophic (acetate-requiring [*ac*]) phenotype (Johnson et al., 2007). The Chl_a fluorescence induction kinetics and charge separation activities were normal in the mutant (Figure 1A, Table 1), indicating no defect in the thylakoid electron transfer chain, which suggested a downstream block in carbon assimilation. Growth on acetate-containing plates was inhibited even at the relatively low light intensity of 40 $\mu E\, m^{-2}\, s^{-1}$ (Figure 1B), and this light-sensitive phenotype was partly alleviated by DCMU, an inhibitor of photosystem II.

The mutant was backcrossed twice to wild-type strains. In the second backcross, 18 tetrads were analyzed, and in each the *ac*, light-sensitive, and antibiotic resistance phenotypes cosegregated in two of the four progeny, indicating a single mutation in a nuclear gene caused by insertion of the cassette. We tested the level of Rubisco, the enzyme in the Calvin-Benson cycle that is responsible for CO_2 fixation. Protein gel blotting revealed that *mrl1* strains lacked both the small and large subunits of the Rubisco enzyme, under conditions both of growth (TAP medium, low light) and of growth arrest (resuspended in MIN medium, high light, 24 h) (Figure 1C). The small subunit is encoded by the *RBCS* family of nuclear genes, while the large subunit is encoded by a single chloroplast gene. By RNA gel blot analysis, we found that *mrl1* strains lacked the *rbcL* mRNA (Figure 2), although its synthesis was unaffected (see below). In all our further phenotypic analyses of the *mrl1* mutant, we found its photosynthetic parameters and light sensitivity indistinguishable from those of classical Rubisco mutants, such as 18-5B (Spreitzer et al., 1985), indicating that the main (or sole) function of MRL1 is to allow accumulation of *rbcL* mRNA. We concluded that the nuclear gene mutated in this strain encodes a protein necessary for maturation/stability of the *rbcL* transcript. In keeping with the accepted nomenclature, we have called the mutated gene *MRL1*.

The *MRL1* Gene Encodes a PPR Protein

An indexed cosmid library was used to complement the *mrl1* mutant as described earlier (Kuras et al., 2007). A cosmid was identified, 21.11G, as capable of restoring phototrophy. It contains a 27.2-kb fully sequenced region of chromosome 6 (5935919-5963122 in genome version 4, corresponding to scaffold_12:1776719-1803922 in version 3). Based on the current annotation, the cosmid insert contains four putative genes, two of which are supported by EST data. Performing different restriction digests on the cosmid and using the digested DNA to transform *mrl1*, we found that the complementing region corresponded to one of the two EST-supported genes, annotated as *PPR2*, which we now rename *MRL1*. A 10.9-kb *Aat*II fragment of the cosmid corresponding to this gene (Figure 3A) was purified and used to retransform a cell-walled *mrl1* strain, to generate the complemented strain *mrl1.C*.

DNA gel blotting was used to analyze the *MRL1* locus in the mutant. Using the enzyme *Xma*I, which cuts at two sites within *MRL1* (Figure 3A; see Supplemental Figure 1 online), we observed that the 4.5-kb fragment corresponding to the 5′ part of the gene was replaced by a 3.2-kb fragment. In a *Nhe*I digest, the 12-kb band corresponding to the 5′ part of *MRL1* was shifted to 7.5 kb in the mutant. Figure 3A shows the putative position of the *aphVIII* gene in *MRL1*.

We obtained and sequenced two cDNA clones derived from *MRL1*. Because similarity with the *MRL1* gene in the closely related alga *Volvox carteri* extended beyond the 5′ end of the longest *Chlamydomonas* cDNA clone, we postulated that it was truncated at the 5′ end. Indeed, using primers designed to amplify the upstream region, we retrieved by RT-PCR a 650-nucleotide extension comprising the beginning of the coding sequence and 57 nucleotides of the 5′ untranslated region (UTR). We found an in-frame stop codon (UAG) 30 nucleotides upstream of the putative start AUG, providing further evidence that we had identified the actual translation start site. New gene models were generated for both species and placed in the gene catalog (ID 206534 for *Chlamydomonas* and ID 127498 for *Volvox* version 1). The *Chlamydomonas MRL1* gene contains 11 exons (Figure 3A). Its coding sequence spans 6901 nucleotides on the genome and 4308 nucleotides on the mRNA. At 1138 nucleotides, the 3′ UTR is unusually long. The nonphototrophic phenotype of *mrl1* could not be complemented by transformation with the reconstituted full-length cDNA, but this was achieved with a chimeric construct containing the promoter and 5′ portion of the gene fused to the cDNA (Figure 3A).

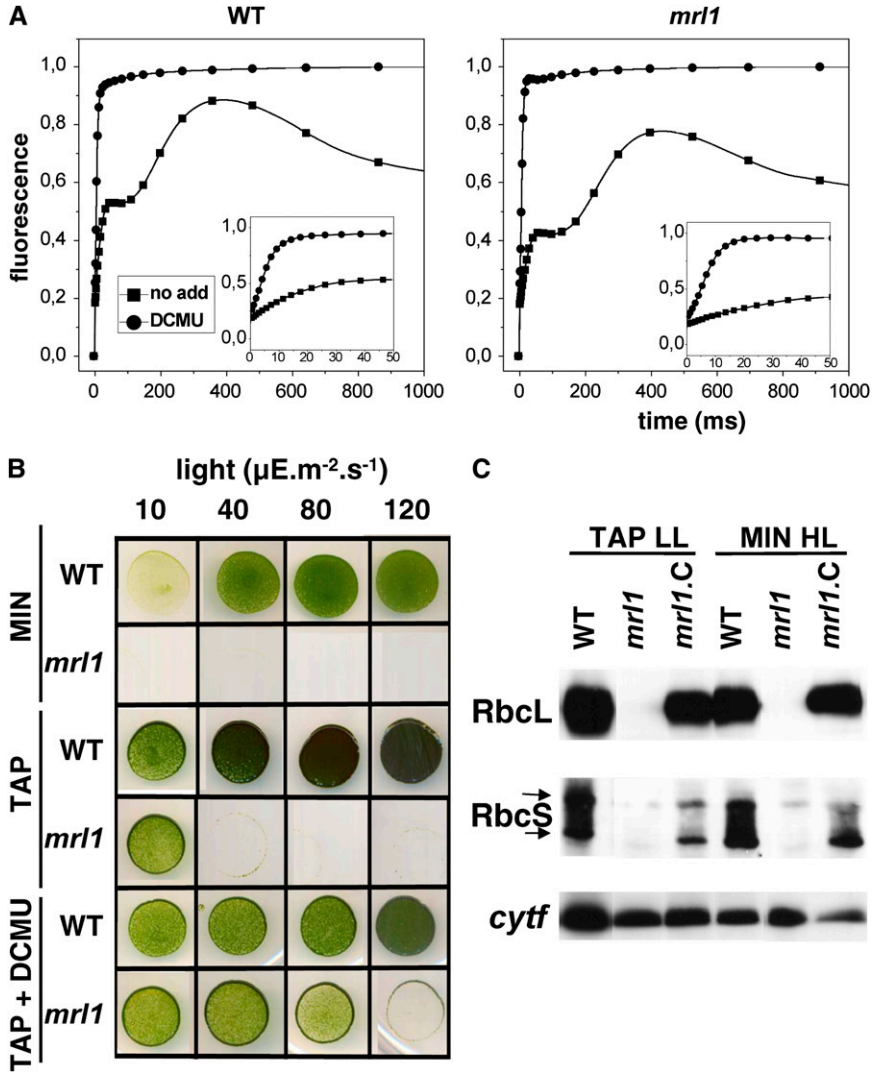

Figure 1. Phenotype of the *mrl1* Mutant.

(A) Fluorescence transients of *Chlamydomonas* wild-type and *mrl1* strains in the presence or absence of DCMU (20 μM). Inset: same traces on an expanded time scale

(B) Wild-type and *mrl1* cells were resuspended in water at a concentration of 10^4 cells mL^{-1} and spotted onto Petri dishes of TAP or MIN media and grown for 10 d. In the last two lines, cells were mixed with DCMU (10 μM final concentration) before spotting onto TAP.

(C) Immunoblot of total cell extracts of wild-type, *mrl1*, and *mrl1*.C (complemented *mrl1* strain) strains reacted with an antibody to RbcL and RbcS (Rubisco large and small subunits) and an antibody to cytochrome *f* (*cytf*) as a control. RbcS appears as a double band (arrows) in this gel system.

[See online article for color version of this figure.]

The hypothetical MRL1 protein is composed of 1435 amino acids with an estimated molecular mass of 138 kD. It can be divided into five regions: a chloroplast-targeting peptide (19 amino acids based on WolfPSORT and ChloroP, corresponding approximately to the region that is not conserved in *Volvox*), an N-terminal region, a PPR domain, a new conserved domain, which we call MRL1-C, and a C-terminal tail rich in Ala and Gly (Figure 3A; see Supplemental Figure 2 online). From amino acids 155 to 593, the TPRpred program at the Max Planck Institute (http://frpred.tuebingen.mpg.de/tprpred) (Biegert et al., 2006) predicts MRL1 to have 10 PPR motifs. Upon further analysis and based on the comparison with other organisms, we found two additional PPR motifs contained within this region (see below). According to predictions by PsiPred (http://www.psipred.net/psiform.html), the protein is highly α-helical, not only the PPR domain but also the N-terminal and MRL1-C domains (see Supplemental Figure 2 online). The tail domain is predicted to be essentially a random coil.

MRL1 belongs to a small family of 11 PPR proteins in *Chlamydomonas* that includes MCA1, a stabilization factor for the *petA* mRNA (Loiselay et al., 2008). In contrast with the other family members, MRL1 shows a high degree of sequence

Table 1. Photosynthetic Parameters of *Chlamydomonas* Wild-Type and *mrl1* Cells Grown in Low Light (10 μE m^{-2} s^{-1}) and Treated or Not in High Light (200 μE m^{-2} s^{-1}) for 16 h

	Wild-Type Low Light	Wild-Type High Light	*mrl1* Low Light	*mrl1* High Light
F_v/F_m	0.68 ± 0.10	0.47 ± 0.11	0.70 ± 0.08	0.37 ± 0.09
PSI	1.00 ± 0.15	1.05 ± 0.22	1.38 ± 0.18	0.66 ± 0.23
PSII	1	0.44 ± 0.11	1.1 ± 0.08	0.43 ± 0.09
NPQ	~0	0.28 ± 0.10	0.29 ± 0.08	0.20 ± 0.11

Cells grown in TAP were harvested in the mid-exponential phase and resuspended in MIN medium at a chlorophyll concentration of ~150 μg mL^{-1}. They were kept in the dark under vigorous shaking before measuring F_v/F_m. Normalized PSI and PSII contents were estimated as described in Methods. Fluorescence was first recorded at 50 μE m^{-2} s^{-1}, and NPQ was evaluated as fluorescence quenching ($F_m - F_{m'}/F_{m'}$) after exposure to saturating (1000 μE m^{-2} s^{-1}) light for 10 min.

conservation with other green photosynthetic eukaryotes (Viridiplantae) (Figure 3B; see Supplemental Figure 2 online). The only other *Chlamydomonas* PPR protein that has a putative ortholog in land plants is HCF152, but sequence conservation is low compared with MRL1. Blast searches in the nonredundant and EST databases and in fully sequenced genomes retrieved full or partial MRL1 sequences from green algae and land plants (chlorophytes and streptophytes), but the gene was not found in other algae (rhodophytes, glaucosystophytes, and heterokonts) or nonphotosynthetic organisms. The moss *Physcomitrella patens* was unusual in showing three MRL1 genes we called MRL1A, -B, and -C. Phylogenetic analysis (Figure 3B; see Supplemental Data Set 1 online) indicates that they have arisen by two successive gene duplication events. Pp-MRL1A and Pp-MRL1B have a pairwise Ks value (rate of synonymous substitutions) of 1.13 (S. Rensing, personal communication), consistent with their being derived from the whole genome duplication that occurred in the moss ~45 million years ago (Rensing et al., 2007).

In MRL1 proteins, the best conserved regions are the PPR and MRL1-C domains (see Supplemental Figure 2 online). Compared with algae, land plants tend to have a longer N-terminal domain and a shorter C-terminal tail. The tail is longest in *Chlamydomonas* and *Volvox*, but complementation experiments using the *mrl1* mutant showed that it is dispensable for function. Indeed, the mutant could be complemented to phototrophy using the promoter-cDNA plasmid restricted with *Sca*I that cuts in the vector part, but also with enzymes that cut within the tail (*Bst*EII, marked as B in Figure 3) or at the end of the MRL1-C domain (*Sgr*AI, marked as S). However, cutting at the *Mlu*I site (marked as M), near the start of the MRL1-C domain, gave no phototrophic colonies, indicating that the C-domain is essential. In keeping with its functional importance, the MRL1-C domain is well conserved, except for three insertions in the *Chlamydomonas* and *Volvox* proteins. BLAST searches with this domain alone found it only in MLR1 proteins.

Transcription of *rbcL* Is Unaffected in *mrl1*

PPR proteins are known as sequence-specific RNA-interacting proteins, participating in a variety of functions, including RNA stabilization and modification (Delannoy et al., 2007). To ascertain that the *mrl1* mutation prevents stabilization of the mRNA rather than its synthesis, we pulse-labeled permeabilized wild-type and *mrl1* cells using [α-^{33}P]UTP for 15 min, and the labeled RNA samples were used as probes in hybridizations to filter-blotted *rbcL*, *psbB*, and *petA* gene fragments. After normalization to the *psbB* transcript, we found no significant difference in the synthesis of *rbcL* between *mrl1* and wild-type strains (Figure 4). We conclude that *rbcL* mRNA transcription is unaffected in the mutant and that its decreased accumulation is due to an enhanced degradation compared with the wild type. Using RT-PCR, we found traces of the *rbcL* mRNA in the *mrl1* mutant, especially in conditions of growth arrest (see Supplemental Figure 3B online). We used RNA ligation–mediated rapid amplification of cDNA ends (RLM-RACE) to identify the 5′ end of the mRNA transcript and found a single 5′ end (see Supplemental Figure 3C online). Treatment with pyrophosphatase increased signal intensity, indicating that this 5′ end is triphosphorylated and corresponds to a transcription start site. Its abundance was severely reduced in the mutant, but its sequence was unchanged, indicating that loss of MRL1 does not induce abnormal processing of the transcript.

We also examined the 76-5EN mutant (Hong and Spreitzer, 1994), which was described as being deficient in *rbcL* transcription. Preliminary genetic analysis suggested that it is allelic or closely linked to *mrl1* (no segregation in 16 zygotes examined), even though its *MRL1* locus does not appear rearranged in DNA gel blots. Because preliminary experiments with the original 76-5EN strain showed a low efficiency of transformation, we crossed it to the wild type and generated strain 76.5EN.1B, which could be transformed with the promoter-cDNA fusion

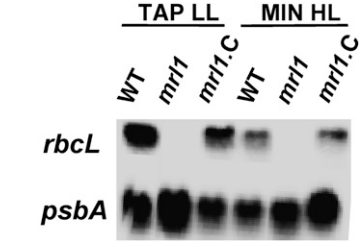

Figure 2. Total Cell RNA Hybridized with *rbcL* and *psbA* as a Control Probe.

The *mrl1* strain accumulates no *rbcL* mRNA compared with the wild type or *mrl1*.C. Low light is 10 μE m^{-2} s^{-1}, and high light is 200 μE m^{-2} s^{-1}.

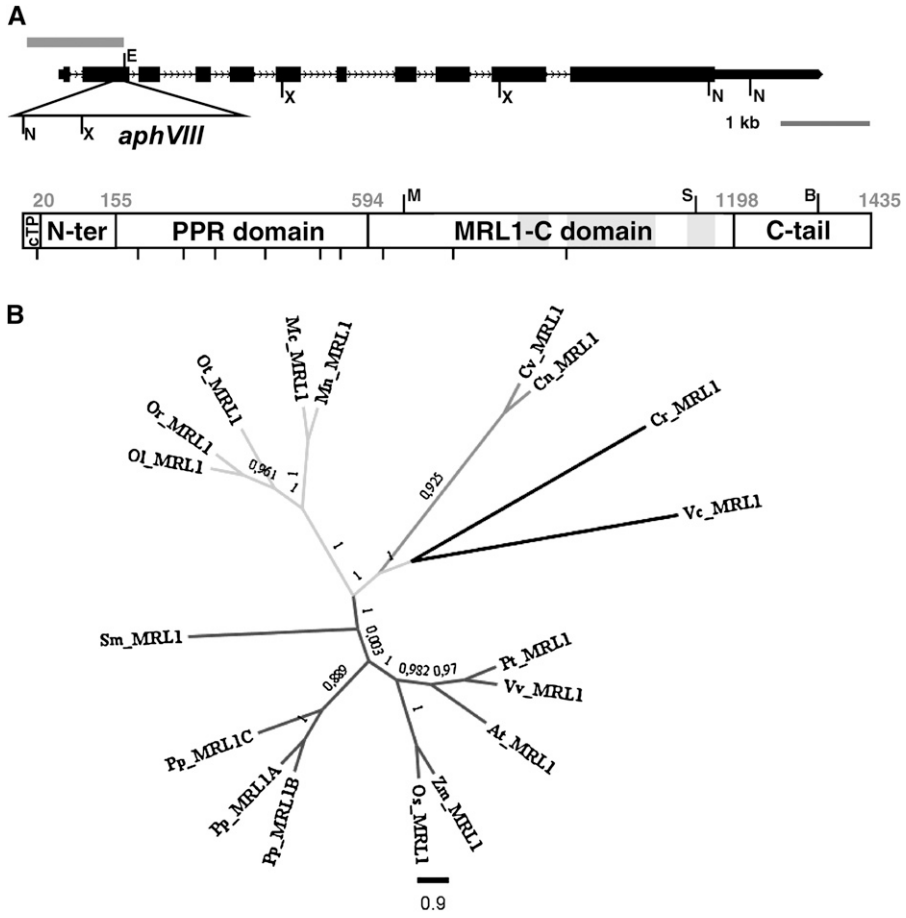

Figure 3. The *MRL1* Gene.

(A) Top: Map of the *MRL1* gene. Location of the *aphVIII* gene is indicated, together with the *Nhe*I (N) and *Xma*I (X) sites used in DNA gel blotting (see Supplemental Figure 1 online). Exons are shown as boxes and introns as arrowed lines. The gray bar at the 5′ end denotes the genomic fragment that was cloned into the *Eco*RI (E) site of the cDNA to generate the promoter-cDNA construct. Bottom: Map of the MRL1 protein, showing its transit peptide (TP) and four domains (with numbering of their first amino acid) and the positions of introns (ticks on lower line) and of restriction enzyme sites used in transformation experiments (M, *Mlu*I; S, *Sgr*AI; B, *Bst*EII). The three insertions found in the C-domain of *Chlamydomonas* compared with other sequences are indicated by gray shading.

(B) Cladogram of MRL1 proteins. The tree was obtained with the program PhyML, based on the alignment of Supplemental Figure 2 online after truncation of the first 589 ill-aligned positions. Streptophytes appear at the bottom (*Sellaginella*, *Physcomitrella*, rice, maize, *Arabidopsis*, grapevine, and *Populus*), Prasinophytes at top left (*Ostreococcus* and *Micromonas*), and other chlorophytes at top right (*Chlorella*, *Volvox*, and *Chlamydomonas*). Each branch is labeled with its aLRT value.

construct. This strain was efficiently complemented by the *Sca*I-restricted construct, restoring a photosynthetic phenotype with an efficiency similar to that observed in *mrl1* strains. We conclude that 76.5EN is mutated in the *MRL1* gene and designate this allele as *mrl1-2*.

MRL1 Is Localized to the Stroma and Is Part of a High Molecular Mass Complex Profoundly Affected by the Absence of Its RNA Target

An antibody against MRL1 detected a protein of around 120 kD in chloroplast stromal extracts from the wild-type strain, but not from the *mrl1* mutant (Figure 5A). To test whether MRL1 forms part of an RNA/protein complex, chloroplast stroma was prepared and high molecular mass complexes were separated by size exclusion chromatography with or without prior treatment by RNase I. As shown in Figures 5B and 5C, the peak of MRL1 elution was in fraction 5, corresponding to an approximate molecular mass of ~800 kD. After treatment with RNase I, however, complex size was reduced to ~600 kD (fraction 7), suggesting that the complex contains an RNA moiety. To test whether the *rbcL* mRNA was part of this complex, we generated a chloroplast transformant where the entire *rbcL* gene was deleted. When isolated stroma fractions were examined, the peak of MRL1 elution was now in fraction 8, corresponding to a peak in the range of 550 kD. This demonstrates that the MRL1 protein

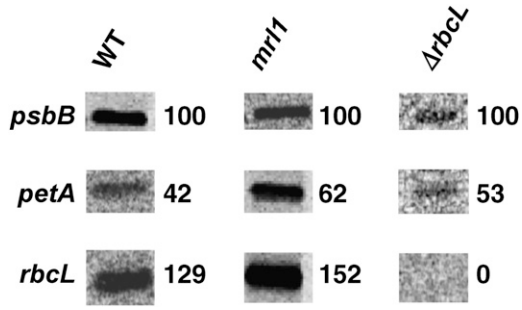

Figure 4. Run-On Transcription Experiment.

In vivo–labeled RNA from wild-type, *mrl1*, and Δ*rbcL* strains was hybridized to gene fragments separated by electrophoresis and blotted onto nitrocellulose. Numbers indicate labeling intensity, normalized to the *psbB* control. Specificity of the *rbcL* signal is indicated by its absence in the Δ*rbcL* strain.

interacts with the *rbcL* mRNA in vivo to form a high molecular mass complex that may or may not include other proteins.

The 5′ Region of the *rbcL* Transcript Is the Target of MRL1

To establish which part of the *rbcL* transcript interacts with MRL1, we introduced into the chloroplast genome chimeric constructs containing a reporter gene fused to either the 3′ or the 5′ UTR of the *rbcL* gene. When transformed into *mrl1*, the *5′atpA-aadA-3′rbcL* resistance cassette (Goldschmidt-Clermont, 1991) yielded the same number of spectinomycin/streptomycin-resistant transformants as when transformed into the wild-type control. This indicates that the chimeric *5′atpA-aadA-3′rbcL* transcript is stable in an *mrl1* background and, hence, that the target of MRL1 is not the 3′ UTR of *rbcL*. By contrast, a construct carrying the *petA* reporter gene under control of the *rbcL* 5′ region (pRF) was unable to express cytochrome *f* when transformed into an *mrl1* strain. Transformants obtained in a wild-type background showed normal fluorescence induction kinetics (Figure 6A), indicating that the *rbcL* 5′ region is able to drive *petA* expression, but those created in the *mrl1* background showed induction kinetics typical of cytochrome b_6f mutants. This result was confirmed by RNA gel blot analysis (Figure 6B), which showed that the absence of MRL1 prevents accumulation of the *rbcL-petA* chimeric transcript. We conclude that the 5′ UTR is the site of interaction between MRL1 and the *rbcL* transcript.

Analysis of the PPRs in MRL1

Because target recognition is believed to be mediated by the PPR repeats, we examined in detail the sequence alignment of the PPR domain and asked whether the repeats themselves

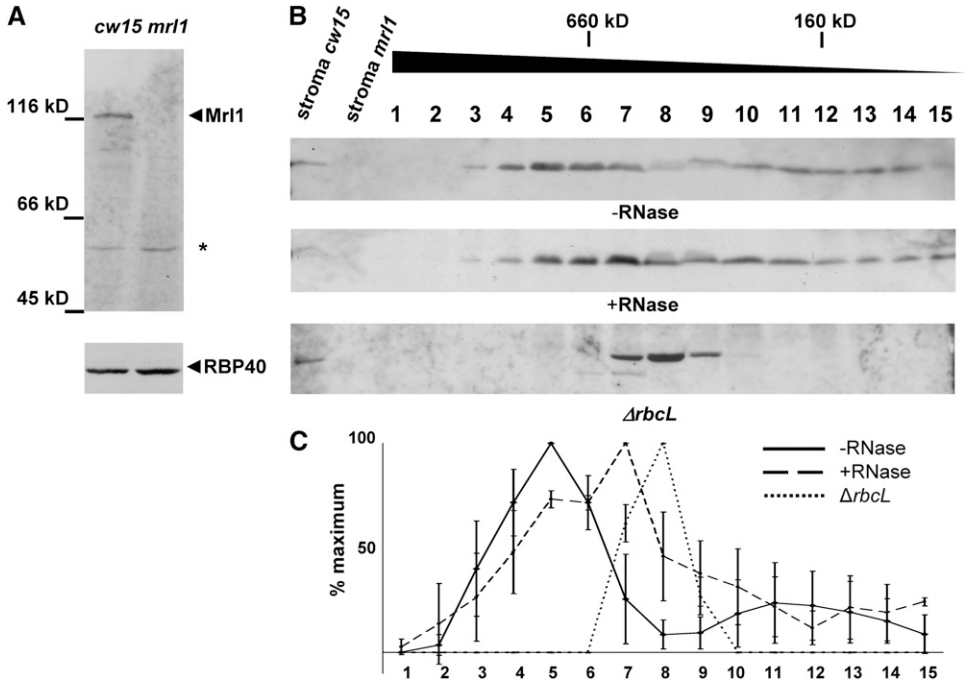

Figure 5. A High Molecular Mass Ribonucleoprotein Complex Containing MRL1 Is Located in the Chloroplast Stroma in *Chlamydomonas*.

(A) Detection of MRL1 in stromal proteins from *cw15* and *mrl1* by immunoblotting using the MRL1 antibody. A cross-reacting protein at 55 kD is marked by an asterisk. The *psbD*-specific translational activator RBP40 was used as a loading control.

(B) Stromal proteins were separated by size exclusion chromatography, and fractions 1 to 15 were subjected to protein gel blot analysis using the MRL1 antibody. Samples are from *cw15* (treated or not with 250 units RNase I) and from the *cw15 ΔrbcL* strain. Molecular masses were calculated by parallel analysis of high molecular mass calibration markers.

(C) Quantitation of signal intensity in **(B)**, with error bars calculated from three (the wild type and *mrl1*) or two (Δ*rbcL*) independent experiments.

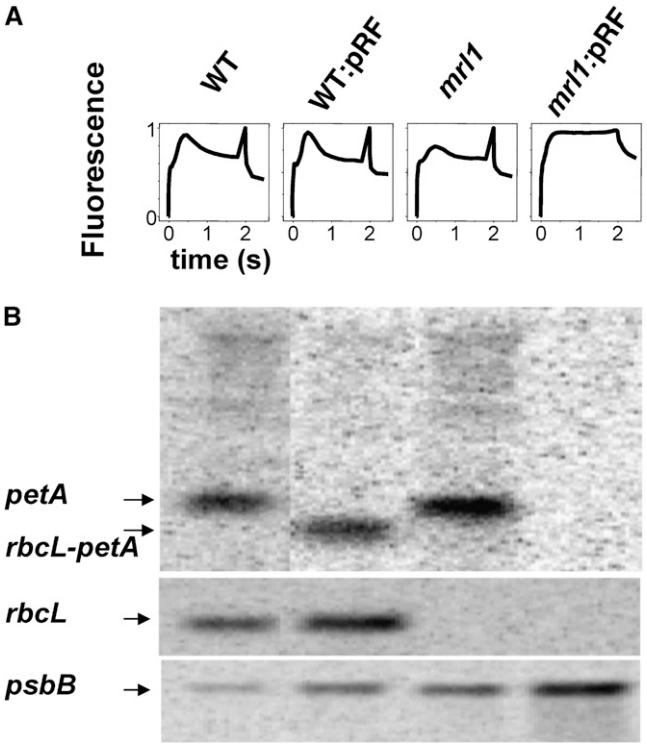

Figure 6. MRL1 Targets the 5′ Region of *rbcL*.

(A) The wild type, *mrl1*, and the corresponding pRF-transformed strains containing the *5′rbcL-petA* chimera replacing the native *petA* gene were analyzed by fluorescence induction to measure photosynthetic activity. Actinic light is turned on at t = 0 and a pulse of saturating light superimposed at t = 2 s to reach F_m, after which the light is turned off. Curves are normalized to F_m.

(B) RNA gel blot hybridization analysis of the strains in **(A)**, showing accumulation of either endogenous or chimeric *petA* transcript and *rbcL* mRNAs; *psbB* was used as a loading control.

were conserved. After refining the alignment based on the predictions of TPRpred (see Supplemental Figure 2 online), we identified 12 repeats in most MRL1 proteins, but only 11 in *Physcomitrella* MRL1C and 14 in the proteins from prasinophyte algae (*Ostreococcus* and *Micromonas*). Many of the repeats initially had escaped detection by the TPRpred program, usually because they contain short insertions between the two α-helices or after the second one, and TPRpred works on a fixed window of 35 residues. However, sequence conservation around the insertions unambiguously defines these regions as PPR repeats, albeit of noncanonical length. Allowing short insertions in the PPR motif helps reconcile sequence alignment and PPR prediction in other algal PPR proteins as well. For some of the repeats, sequence alignment also indicated short deletions. For example, the region in land plants that aligns with PPR#1 in algae has only 34 residues, and PPR#2 has deletions or insertions in most species. These may thus not be bona fide PPRs in all species, yet their sequence characteristics and presence in a PPR domain indicate that they derive from, and probably have kept some of the structural/functional properties of, a PPR.

Using these 224 refined repeats (see Supplemental Data Set 2 online) as independent input sequences, phylogenetic trees were built (see Supplemental Figure 4 online) to determine if the MRL1 protein had been subject to rearrangements within its PPR domain. Overall, the trees show a strong tendency to cocluster the repeats from a given location in the protein, suggesting a largely linear evolutionary history of the PPR domain.

The *Arabidopsis* MRL1 Ortholog Also Acts on the *rbcL* Transcript but Is Not Essential for Photosynthesis

According to Genevestigator (https://www.genevestigator.ethz.ch/gv/index.jsp), the *Arabidopsis* ortholog of *MRL1* is expressed in all green parts, including stems, leaves, and sepals, but not in nongreen tissues, suggesting an involvement in photosynthesis. We analyzed two *Arabidopsis* T-DNA insertion lines, SALK-072806 (At-*mrl1-1*) and FLAG_568C09 (At-*mrl1-2*), carrying insertions in the 13th and 10th exons, respectively (i.e., within the PPR domain) (Figure 7A). Homozygous plants carrying the At-*mrl1-1* and At-*mrl1-2* mutations were genotyped and confirmed by RT-PCR to lack the *MRL1* transcript (Figure 7B). As controls, we used, respectively, the Columbia-0 (Col-0) line and *MRL1-2*, a wild-type progeny from an At-*mrl1-2* heterozygous stock in the Wassilewskija background. Whether sown on sucrose-supplemented Murashige and Skoog medium or on soil (Figure 7C), neither growth nor color phenotype was observed, even in short-day conditions (8 h light/16 h dark).

Accordingly, functional analysis of mutant *Arabidopsis* plants did not show any significant change in the photosynthetic process compared with the wild type. For example, the photosystem II (PSII) to photosystem I (PSI) ratio (Figure 8A), which was assessed in planta from their relative contribution to the light-induced electrochromic signal, was not affected. The fluorescence parameter ΦPSII, which directly measures PSII-driven electron flow, was unchanged (Figure 8B). Furthermore, RNA gel blots did not detect any alterations with probes directed against *psbA*, *psbB*, *petB*, *psbD*, *psbN*, *atpBE*, *psbEL*, *psaA*, *psaC*, *petA*, *atpA*, *atpF*, *ndhB*, or *ndhK* (see Supplemental Figure 5 online). Because of the observed lack of *rbcL* transcript accumulation in *Chlamydomonas*, we tested *rbcL* mRNA accumulation levels in the plant mutant lines (Figure 8C). While two *rbcL* transcripts can be resolved by gel blot analysis in wild-type lines (lanes Col-0 and *MRL1-2*), the shorter transcript appeared to be completely absent in the mutants, a result that was confirmed (Figure 9) by the more sensitive techniques of primer extension and RLM-RACE. The size and 5′ sequence of the shorter mRNA that is missing in the mutant are similar to those reported by Shiina et al. (1998) for the processed *rbcL* transcript in various land plants. In the mutant, the amount of the longer transcript appeared unaffected. The accumulation of the Rubisco protein was only marginally affected (see Supplemental Figure 6A online). Polysome gradient analysis (see Supplemental Figure 6B online) showed that the remaining mRNA was loaded into the heavy fractions. Still, we observed a small but reproducible shift of *rbcL* mRNA toward lighter fractions in the mutants, not seen in the *atpB* control.

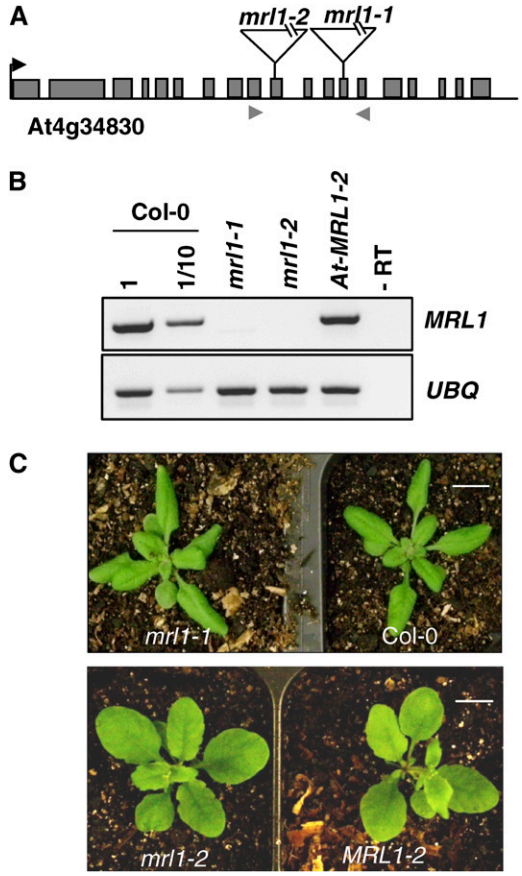

Figure 7. Analysis of At-*mrl1* Mutants.

(A) Sites of T-DNA insertions in At-*MRL1*. The arrowheads indicate primers used for the RT-PCR shown in **(B)**. Gray boxes, exons; black lines, introns.

(B) RT-PCR was performed using 33 cycles for *MRL1* and 27 cycles for *UBQ*.

(C) Plants of the indicated genotypes were grown on soil for 3 weeks under a 16-h-light/8-h-dark photoperiod. The morphological differences can be ascribed to the fact that *mrl1-2* is in the Wassilewskija ecotype and *mrl1-1* is in Col-0. (*MRL1-2* is a wild-type progeny from the heterozygous *mrl1-2* seed stock.) Bar = 1 cm.

[See online article for color version of this figure.]

In *Chlamydomonas*, Impairment of Electron Flow from H_2O to CO_2 Is Compensated for by an Increased Capacity to Reduce Molecular Oxygen

As mentioned above, the fluorescence induction profile of the *Chlamydomonas mrl1* mutant was similar to that of the wild type (Figure 1A). In both strains, the pseudo-steady state fluorescence level (F_s) was well below the maximum level (F_m), suggesting that the rate of electron flow is similar in the two strains at steady state and close to that of PSII turnover. From the kinetics observed in the presence of DCMU (Butler, 1978), the latter was estimated to be around 10 ms in both strains (Figure 1A, inset). Thus, the absence of Rubisco does not represent a major bottleneck in electron transfer downstream of PSII.

A similar conclusion was reached when electron flow was assessed under continuous illumination rather than during a dark-light shift (Figure 10A). The parameter ΦPSII, which directly measures PSII-driven electron flow, was only marginally decreased in the *mrl1* mutant as opposed to the Δ*petB* mutant (Kuras and Wollman, 1994), which lacks cytochrome b_6f and was used in the experiment as a control for impairment of electron transfer. Conversely, oxygen evolution that reflects electron flow from H_2O to terminal acceptors was largely suppressed in the *Chlamydomonas* mutant at all light intensities tested (Figure 10B), in line with its inability to grow autotrophically. Because electron flow is not accompanied by a commensurate net O_2 evolution, the final electron acceptor must be molecular oxygen itself.

The chlororespiratory process (reviewed in Peltier and Cournac, 2002), whereby plastoquinol is oxidized by O_2 through the action of the plastoquinol terminal oxidase, is not likely to account for such a high rate of electron transport. Indeed, we found that propylgallate, a known inhibitor of plastoquinol terminal oxidase, had a similar effect on ΦPSII in the *mrl1* and wild-type strains. We next considered two other possible routes for oxygen reduction, the so-called Mehler reaction (Mehler, 1951), whereby

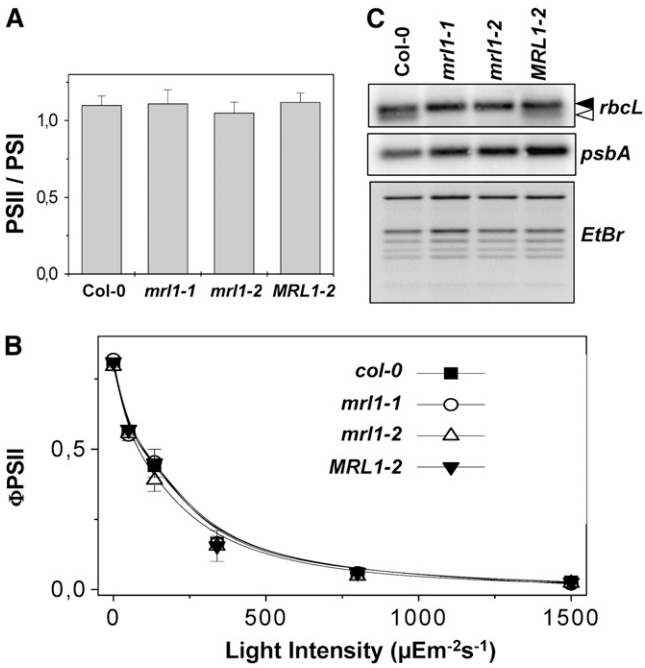

Figure 8. Analysis of At-*mrl1* Mutants.

(A) PSII-to-PSI ratio, measured from their respective contribution to the light-induced electrochromic shift (ECS) signal. Bars represent the SE of three measurements

(B) Light-driven electron transport activity, as derived from the fluorescence parameter ΦPSII (see Methods). Bars represent the SE of three measurements

(C) RNA gel blot analysis of *rbcL* and *psbA* mRNA. The bottom panel is the ethidium bromide–stained gel. The closed arrowhead indicates the primary transcript and the open arrowhead the processed species.

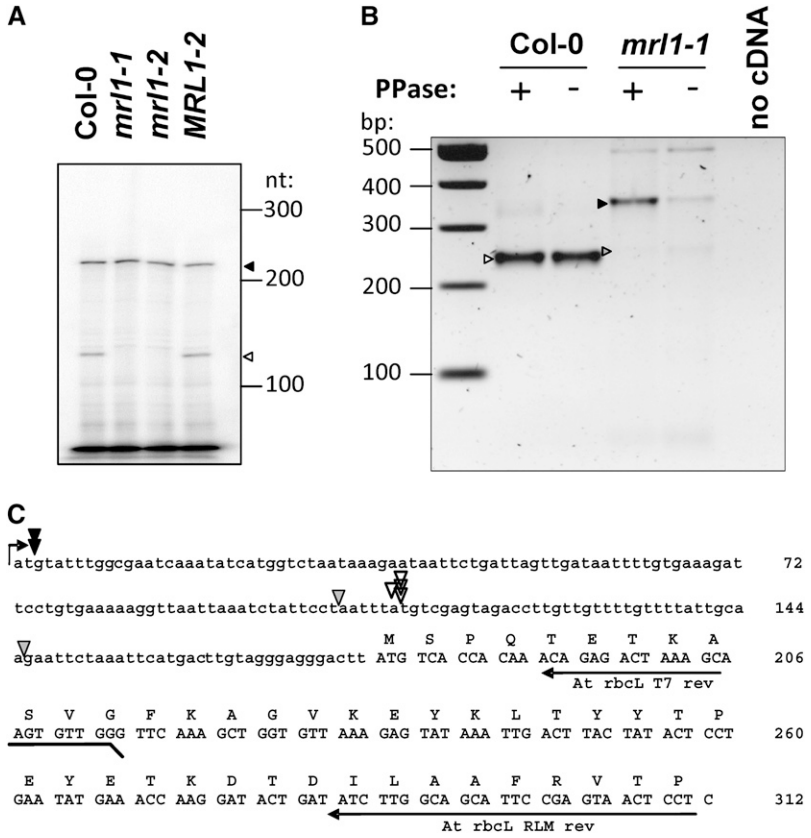

Figure 9. Analysis of At-*mrl1* Mutants.

(A) Primer extension analysis of the *rbcL* transcript, showing absence of the processed transcript (open arrowhead) in the mutants but retention of the primary transcript (closed arrowhead).

(B) and **(C)** RLM-RACE experiments: result of the PCR step and alignment on the *rbcL* upstream sequence of the 5′ ends identified by cloning the bands marked by arrowheads. After ligation of an RNA oligonucleotide at its 5′ end, the mRNA was reverse transcribed and amplified. In the mutant, a single transcript was amplified (closed arrowhead), and its enhancement by pyrophosphatase (PPase) treatment indicates that it corresponds to a primary transcript. In the wild type, for reasons unclear, only the shorter form was amplified (open arrowhead). Its lack of enhancement by PPase treatment indicates a 5′-monophosphate end typical of a processed RNA. In the mutant, only a very faint band (gray arrowhead) was found at this position, but its sequencing variable 5′ ends, all different from that of the processed transcript in the wild type.

photosynthesis reduces molecular oxygen at the acceptor side of PSI, and the malate shuttle, which allows consumption of photosynthetically generated reducing equivalents by respiration in the mitochondrion (Edwards and Andreo, 1992). To distinguish between these two pathways, we measured ΦPSII and respiration rate simultaneously in a sealed cuvette, as a function of the decreasing oxygen concentration (Figure 11). At 10 μM O_2, ΦPSII was decreased by 90% in the mutant compared with only ~30% in the wild type, while respiration was unaffected, in line with its known low K_m (3 μM; Forti and Caldiroli, 2005). The low affinity of ΦPSII for O_2 (30 to 40 μM) in *mrl1* was comparable to that of the Mehler reaction (~25 μM; Forti and Caldiroli, 2005), suggesting that this reaction is responsible for accepting electrons downstream of PSI in the mutant.

We then analyzed the effect of continuous high light on the electron transfer chain in *mrl1*. After 16 h at 200 μE m^{-2} s^{-1} (high light), both the wild type and *mrl1* strain suffered a drop in PSII activity, reflecting photoinhibition (Table 1). However, reduced PSII activity did not result in a major imbalance in photosynthesis in *mrl1* because PSI activity was also decreased to nearly the same extent. This PSI photoinhibition was not observed in the wild type. Interestingly, PSI activity recovered more slowly than PSII in the mutant (see Supplemental Figure 7 online), indicating that repair of light-induced damage was less efficient for PSI than for PSII. Furthermore, nonphotochemical quenching of absorbed energy (NPQ), a physiological response to light stress, was observed in *mrl1* grown in low light (Table 1), while prior exposure to high light is necessary in the wild-type strain (Niyogi, 1999). Finally, despite the growth arrest in high light, no extensive cell death was observed in *mrl1* cultures, unless they were deprived of oxygen during light exposure (Table 2). This stands in stark contrast with a Δ*psaB* mutant devoid of PSI, where oxygen deprivation rescues the strain (Table 2). These observations suggest that the Mehler reaction plays an important photoprotective role in the *Chlamydomonas mrl1* mutant, in spite of the fact that it produces potentially harmful reactive oxygen species (ROS).

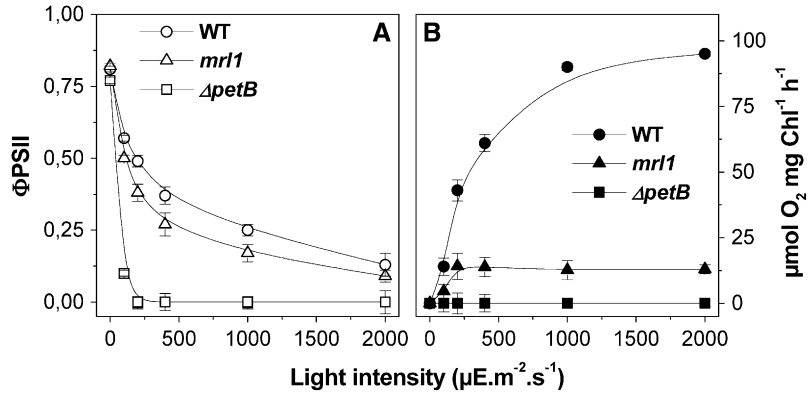

Figure 10. Light Dependence of ΦPSII and O_2 Evolution in *Chlamydomonas*.

Light dependence of ΦPSII **(A)** and O_2 evolution **(B)**. Chlorophyll concentration was ~150 µg mL^{-1}. O_2 evolution was followed with a Clark electrode by increasing light intensity every 2 min and expressed as net photosynthesis (i.e., the maximum rate of photosynthesis after correction for respiration) at any given light intensity. Bars represent the SE of three independent experiments.

DISCUSSION

In this article, we show that MRL1 contributes to the stability and/or maturation of the *rbcL* transcript both in *Chlamydomonas* and in *Arabidopsis*. In the alga, inactivation of *MRL1* completely prevents accumulation of the mRNA and of the Rubisco protein, while in the plant, only a very slight reduction in Rubisco content is observed. This leads to dramatic differences in growth phenotypes, nonphototrophic and light-sensitive in the case of the alga, normal in the plant.

The Cr-*mrl1* Mutant: How to Survive without Rubisco

Upon illumination, the *Chlamydomonas* mutant performs a sustained H_2O-H_2O cycle, likely involving the Mehler reaction, superoxide dismutase, and catalase. The Mehler reaction leads to ROS accumulation in high light (i.e., when O_2^- is not efficiently scavenged by superoxide dismutase and catalase activities) (Ort and Baker, 2002), a possible explanation for the light-sensitivity of the mutant and its alleviation by DCMU. ROS may induce photoinhibition of PSII and cause direct photodamage to PSI, as already proposed (Munekage et al., 2002). The nature of the PSI damage is unknown, but its slow recovery suggests that it is severe. Yet, cell death was not observed, unless oxygen was removed (Table 2). This stands in sharp contrast with the behavior of PSI mutants, which also show a high photosensitivity but are rescued by anaerobiosis. Thus, in spite of its negative effects on the photosystems, the Mehler reaction may play a protective role when CO_2 fixation is impaired. By permitting electron flow even when PSI acceptors are reduced, the Mehler reaction allows for the generation of a light-induced $\Delta\mu_H^+$. This not only triggers NPQ-mediated photoprotection (Table 1), but may also allow residual ATP synthesis, contributing to cell survival.

Molecular Target and Mechanism of Action of a Conserved PPR Protein

PPR proteins are present in most eukaryotic phyla but are exceptionally numerous in land plants. A rapid diversification of the family has occurred between the colonization of terrestrial habitats and the monocot-dicot divergence (O'Toole et al., 2008), possibly as a means to suppress deleterious mutations in the organellar genomes (Maier et al., 2008). Thus, even though PPR proteins in land plants are often well conserved, the high degree of sequence similarity observed between algal and plant MRL1 (see Supplemental Figures 2 and 4 online) is highly unusual. It suggests not only that the mechanism of action of MRL1 has been largely conserved throughout evolution of the green lineage, but that its target has been fixed early on.

Indeed, our functional and molecular analyses in two model species have yielded no indication that MRL1 would have a target other than the *rbcL* mRNA. Our reporter gene experiments in *Chlamydomonas* (Figure 6) show that the molecular target of MRL1 lies upstream of the *rbcL* coding sequence (nucleotides −161 to −1 with respect to the translation start site), in a region that includes both the promoter and the 5′ UTR. Because run-on experiments show that the mutation does not affect *rbcL*

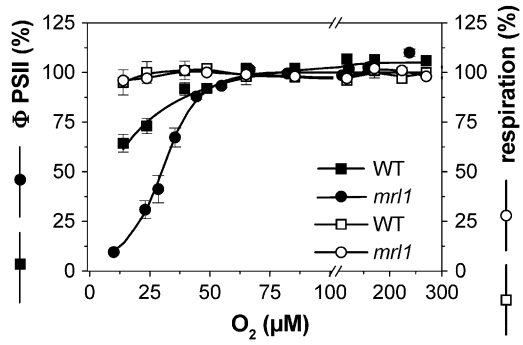

Figure 11. ΦPSII and Respiration as a Function of Oxygen Concentration in *Chlamydomonas mrl1* versus Wild-Type Cells.

Oxygen concentration was varied by letting the cells respire in the dark. Cells were illuminated for 30 s before measuring the ΦPSII at 130 µE m^{-2} s^{-1}. Bars represent the SE of three independent measurements.

Table 2. *Chlamydomonas* Cell Survival in MIN Medium at 200 μE m^{-2} s^{-1}

	Wild Type		*mrl1*		*ΔpsaB* (PSI-Less)	
	O_2	$-O_2$	O_2	$-O_2$	O_2	$-O_2$
24 h	99.9	95	95	74	2	90
48 h	96	96	74	28	0	85

Cells were grown in TAP medium, centrifuged and resuspended to a concentration of 10^6 cells mL^{-1} in MIN media with $NaHCO_3$ (5 mM), and exposed to air ($+O_2$) or nitrogen ($-O_2$). For counting, cells were first stained with Alcian blue (which stains only dead cells) and then with iodine (which stains and immobilizes all cells), and percentage of survival was calculated.

transcription, we propose that MRL1 stabilizes the *rbcL* mRNA by binding to its 5′ UTR (nucleotides −92 to −1). As a possible mechanism, it could act via stabilization of the secondary structures (Suay et al., 2005) known to shield the RNA from degradation by endonucleases. Direct binding to the target has been proposed or demonstrated for several mRNA stabilization (M) factors in *Chlamydomonas* (Boudreau et al., 2000; Herrin and Nickelsen, 2004; Loiselay et al., 2008) and for other PPR proteins (Nakamura et al., 2003; Schmitz-Linneweber et al., 2005; Pfalz et al., 2009). In support of this hypothesis, we found that MRL1 is part of a high molecular mass ribonucleoprotein complex, whose size is shifted by RNase I treatment. We note that the size of the MRL1 complex is less affected by the RNase treatment than by a total lack of *rbcL.* This could suggest that the MRL1 protein protects a region of the *rbcL* mRNA from the action of the RNase.

In accordance with previous studies (Dron et al., 1982; Anthonisen et al., 2001), we found a single *rbcL* transcript in *Chlamydomonas*, and the presence of a 5′ triphosphate indicates that it is not a processed form (see Supplemental Figure 3 online). It is still detectable in the mutant, consistent with our finding that the mutation prevents the stabilization of the *rbcL* transcript, but not its transcription. Genetic analysis and complementation indicate that the previously described 76-5EN mutation (Hong and Spreitzer, 1994) is in fact an allele of *mrl1*. This casts some doubt on its reported defect in *rbcL* transcription. We note that the RNA run-on transcription experiment reported by these authors shows a substantial labeling of *rbcL* in the mutant, and we propose that the reduced signal they observe is due to rapid degradation of the transcript during the in vivo labeling, rather than transcriptional block. This novel *mrl1-2* allele should prove a valuable tool for further dissection of MRL1 function.

In comparison with *Chlamydomonas*, the less severe phenotype of the *Arabidopsis mrl1* mutants is explained by the coexistence of two *rbcL* transcripts in the latter. In all dicot and monocot species that have been examined in detail, the *rbcL* gene yields a single primary transcript, which is then processed into a shorter form. The At-*mrl1* mutants miss only the processed form, and we conclude that MRL1 is necessary either for processing or for stabilization of the processed form. The mechanism that generates this secondary transcript in plants is unknown, but we favor the hypothesis that MRL1 binding defines the endpoint of nucleolytic degradation of the *rbcL* 5′ end. This would be similar to the recently reported mode of action of another PPR, maize PPR10. This protein serves as a mark on its target mRNAs defining the sites where exonucleases will stop and, thus, the mature 5′ and 3′ ends of the processed forms (Pfalz et al., 2009). A binding of MRL1 to the primary *rbcL* transcript is supported by our finding that the mutation affects the distribution of the transcript in the polysome gradient, although other explanations, such as reduced ribosome loading, absence of other protein factors, or enhanced cotranslational mRNA degradation, should also be considered. In any event, while MRL1 certainly plays a more complex role in *Arabidopsis* than in *Chlamydomonas*, the basic mechanism could be very similar. After binding to the primary transcript, MRL1 might either fully stabilize it (as in *Chlamydomonas*) or serve as a mark to determine the endpoint of its nucleolytic processing (as in *Arabidopsis*). In both cases, it could also recruit other factors promoting mRNA stabilization or translation (McCormac et al., 2001). In plants, the primary transcript is intrinsically more stable than in *Chlamydomonas*, and MRL1 is no longer necessary for Rubisco biogenesis. Yet, the resulting increase in *rbcL* mRNA accumulation or the ability to differentially regulate the two mRNAs might benefit the fitness of the organism. This could explain why *rbcL*'s interaction with MRL1 has been retained despite the considerable divergence of its 5′ UTR in terms both of length and sequence.

Can we identify the *cis*-acting determinants of target recognition in *rbcL*, in other words, the MRL1 binding site, by virtue of their sequence conservation? In chlorophyte algae, the *rbcL* 5′ UTRs are too divergent for automatic sequence alignment (see Supplemental Figure 8 online) and no convincing conserved region emerges. The stability sequence described in the 5′ UTR of *Chlamydomonas rbcL* around position −47 (Suay et al., 2005) is conserved only in *Volvox*, which makes it an unlikely candidate for the MRL1 binding site.

By contrast, after aligning *rbcL* 5′ UTR from a variety of streptophytes (see Supplemental Figure 9 online), we found remarkable sequence conservation at the very 5′ end of the processed transcript, the hypothesized MRL1 target in *Arabidopsis*. The 15-nucleotide consensus sequence URUCGAGYAGACCYY was almost perfectly conserved just downstream of the processing site. In plants, this 15-nucleotide sequence is not predicted to be part of a stable stem-loop structure, and we assume that MRL1 will bind this region in an extended conformation. Similarly, the binding region of MCA1 has been found to lie in the first 21 nucleotides of the *petA* mRNA (Loiselay et al., 2008).

METHODS

Standard nucleic acids manipulations were performed according to Sambrook et al. (1989). Primers are listed in Supplemental Table 1 online.

Chlamydomonas reinhardtii

Strains of *Chlamydomonas* were grown in Tris-acetate-phosphate or minimum (acetate-free) medium, under continuous light (Harris, 1989). For the description of recipient strain XS1 (*cw15 arg7 mt+*) and the

production and complementation of mutants, refer to Johnson et al. (2007) For cell-walled strains, transformation by electroporation was performed at 1 kV (Raynaud et al., 2007) instead of 0.72 kV. cDNA clones were obtained through the Kazusa DNA Research Institute. Crosses were performed using the standard protocol (Harris, 1989) with strains WTS34 +, WT24−, or *cw15 mt*−, for generation of the Δ*rbcL cw15* strain.

Run-on transcription was performed for 15 min as by Gagné and Guertin (1992) with modifications (Stern and Kindle, 1993). Gene fragments derived from coding sequences for *psbB* (1200 nucleotides), *petA* (1000 nucleotides), or *rbcL* (550 nucleotides) were amplified by PCR, gel purified (Qiagen), rerun (100 ng) on a 1.4% agarose gel, and transferred to nitrocellulose (Amersham N+) prior to hybridization with the ^{33}P-labeled RNA.

RNA isolation and gel blot analyses were performed as described (Drapier et al., 1998), and, when mRNA was required for cloning the 5′ extremity of *MRL1* cDNA, the Oligotex mRNA mini kit (Qiagen) was used, followed by reverse transcription (TaKara) and PCR amplification (Phusion high fidelity DNA polymerase; Finnzyme) according to the manufacturers' protocols, with primers MRL1-5′.fwd and MRL1-5′.rev. The *MRL1* promoter fragment was amplified with primers MRL1-pro.fwd and MRL1-pro.rev on genomic DNA. For *rbcL*, we used Thermo X reverse transcriptase (Invitrogen) and the *Taq* PCR core kit (Qiagen) with primers *rbcL*_fwd and *rbcL*_rev (57°C, 35 cycles).

To construct the *rbcL-petA* chimeric reporter, the *rbcL* upstream region (nucleotides −70 to +92 relative to the transcription start) was amplified using primers Cr_P*rbcL* fw and Cr_P*rbcL* rev and cloned into pGEMTeasy (Promega). The *Cla*I-*Nco*I fragment was then inserted into *Cla*I-*Nco*I–digested paAFFF (Wostrikoff et al., 2004), thereby fusing the *rbcL* promoter region to the *petA* coding sequence, generating pRFFF. A 2.9-kb *Sac*I-*Kpn*I *aadA* cassette was excised from pEXC (Fisher et al., 1996), blunted using T4 DNA polymerase, and then subcloned into *Hinc*II-digested pRFFF, yielding pRFFFiK, where the cassette is transcribed opposite to the *rbcL-petA* chimera. For the generation of the Δ*rbcL* strain, the R15 region according to the Rochaix nomenclature was cloned into pUC19 plasmid. The 7459-bp *Ale*I-*Bse*RI fragment from plasmid R15pUC-2 was ligated with the 1940-bp *Sma*I-*Eco*RV fragment from plasmid pMGS, containing the *5′atpA-aadA-3′rbcL* cassette. Both fragments were pretreated with T4 DNA polymerase to generate blunt ends before cloning. In the resulting plasmid, pK15-2, the *aadA* gene is read on the opposite strand relative to *rbcL*.

Chlamydomonas was transformed using tungsten particle bombardment (Boynton and Gillham, 1993), as described by Kuras and Wollman (1994). Transformants were selected on TAP-spectinomycin (100 $\mu g \cdot mL^{-1}$) under low light (5 to 6 $\mu E\ m^{-2}\ s^{-1}$) and subcloned on 500 $\mu g \cdot mL^{-1}$ spectinomycin to reach homoplasmy. Proper insertion of transforming DNA and homoplasmy were checked by PCR.

Protein analysis (Figure 1C) was performed according to Kuras and Wollman (1994). For analysis of high molecular mass complexes (Figure 5), chloroplasts isolated from *cw15* strains according to Zerges and Rochaix (1998) were lysed in nonreducing hypotonic solution (10 mM EDTA, 10 mM Tricin-KOH, pH 7.5, and Roche CompleteMini protease inhibitors). Insoluble material was removed by centrifugation on a 1M sucrose cushion (100,000*g*, 30 min) and the stroma-containing supernatant concentrated in Amicon Ultra filtration devices (Millipore) at 4°C, with or without 250 units of RNaseOne (Promega). Samples (2.5 mg protein) were loaded through an SW guard column onto a 2.15 × 30-cm G4000SW column (Tosoh), and elution was performed at 4°C with buffer containing 50 mM KCl, 5 mM $MgCl_2$, 5 mM ε-aminocaproic acid, and 20 mM Tricin-KOH, pH 7.5, at a pressure of 0.95 MPa. Elution fractions were concentrated using Amicon Ultra devices and subjected to immunoblotting. The Cr-MRL1 antibody was a kind gift of Christian Schmitz-Linneweber and was generated using a 123–amino acid fragment of the MRL1-C domain, outlined in Supplemental Figure 2 online. The stromal loading control RBP40 was used as described by Schwarz et al. (2007).

Spectroscopy, Fluorescence, and Oxygen Measurements

In vivo spectroscopy was performed with a JTS spectrophotometer (Biologic). PSI and PSII contents were calculated from changes in the amplitude of the fast phase (100 μs) of the electrochromic signal (at 520 to 545 nm) upon excitation with a saturating laser flash, as previously described. PSII and PSI contribution were evaluated from the amplitude of the signal measured in the presence or absence of the PSII inhibitors DCMU (20 μM) and hydroxylamine (1 mM) (Joliot and Delosme, 1974).

Fluorescence kinetics were measured using a home-built fluorometer, where fluorescence was excited with a green LED (520 nm) and measured in the near far red. Oxygen evolution was measured using a Clark electrode (Hansatech). ΦPSII, the quantum yield of PSII (Harbinson et al., 1990), was calculated as $(F_{m'} - F_s)/F_{m'}$, where $F_{m'}$ is the maximum fluorescence emission level induced by a pulse of saturating light (~5000 $\mu E\ m^{-2}\ s^{-1}$), and F_s is the steady state level of fluorescence emission. NPQ was calculated as $(F_m - F_{m'})/F_{m'}$, where F_m is the maximum fluorescence (Demmig-Adams et al., 1990). The oxygen dependence of ΦPSII was measured using the JTS spectrophotometer, coupled to a ruthenium oxygen sensor, similar to the one described by Tyystjarvi et al. (1998).

Arabidopsis thaliana

Insertion lines were obtained from the SIGnAL (Alonso and Stepanova, 2003) and FLAGdb (Samson et al., 2002) mutant collections. After vernalization, seeds were germinated either on Murashige and Skoog agar containing 3% sucrose or directly in MetroMix 360 soil under a 16-h-light/8-h-dark photoperiod and fluorescent light. Most experiments were conducted at 200 $\mu E\ m^{-2}\ s^{-1}$, but functional analyses, and some RNA gel blots, were performed at 100 $\mu E\ m^{-2}\ s^{-1}$. Plants were genotyped by PCR. The wild-type allele was amplified using the primer pair LP SALK_072806/RP SALK_072806 for *mrl1-1* and LP FLAG_568C09/RP FLAG_568C09 for *mrl1-2*. The mutant allele was amplified using the forward primers LBb1.3 for *mrl1-1* and LB4 for *mrl1-2* and the specific reverse primers (RPs).

RNA was extracted with Tri-reagent (Molecular Research Center), separated on a 0.8% agarose and 3% formaldehyde gel and blotted onto Genescreen nylon membrane (Perkin-Elmer) by capillary transfer in 25 mM phosphate buffer. Following UV cross-linking in a Stratalinker (Stratagene), membranes were hybridized in modified Church and Gilbert buffer (0.25 M sodium phosphate, 1 mM EDTA, 7% SDS, and 0.1% BSA) with radiolabeled gene-specific probes. The *rbcL* and *psbA* probes were generated by PCR with the Arab *rbcL*.F and Arab *rbcL*.R primers, and At psbA-5′ and At psbA-3′, respectively. For RT-PCR of *MRL1*, 0.5 μg of total RNA was treated with DNase and reverse transcribed using Superscript III (Invitrogen) and used as a template for PCR with primers AtMRL1.fw1/AtMRL1.rev2 or the control primers UBQ1/UBQ2, which amplify UBQ10. DNA was visualized using ethidium bromide.

For primer extension analysis of *rbcL*, 100 nmol of the At*rbcL*rev T7 primer was radiolabeled with [γ-^{32}P]ATP and purified on a Sephadex G-25 column. Five micrograms of total RNA was denatured in a 14.5-μL reaction containing 1× reverse transcription buffer (Promega), 10 nmol deoxynucleotide triphosphate, 40 units RNase inhibitor (New England Biolabs), and 10^5 cpm of radiolabeled primer for 5 min at 75°C and then shifted to 50°C for 5 min. Then, 2.5 units of AMV reverse transcriptase (Promega) was added to the mix, and the reaction was allowed to proceed for another 15 min at 50°C, where it was stopped by adding 7 μL of formamide buffer (98% formamide and 1 mM EDTA). Primer extension products were separated through a 6% polyacrylamide-bisacrylamide (19:1)/7 M urea gel after a 5-min denaturation at 65°C. The gel was then dried and exposed to a phosphor imager screen. RLM-RACE was performed using the Generacer kit (Invitrogen) according to the manufacturer's instruction, with primer AtrbcL RLM rev.

Proteins were extracted from 3- to 4-week-old plants as described (Wostrikoff and Stern, 2007). Ten micrograms of protein was separated through 12% SDS-polyacrylamide gels, transferred onto polyvinylidene fluoride membrane (Perkin-Elmer), and immunodecorated with antibodies against Rubisco LS (Agrisera), PsaD (Agrisera), and cytochrome *f*. ECL Plus (GE Healthcare) was used to reveal the immunoreactive proteins, and the signal was detected using the STORM imager (GE Healthcare).

Spectroscopy and fluorescence measurements were performed using the same setups as described for *Chlamydomonas*. Intact leaves from 4-week-old plants were clamped to the optical instruments using specific sample holders. Humidified air was blown over the leaves during the experiments to avoid CO_2 limitation of photosynthesis.

Phylogenetic Analysis

The refined Clustal alignment of Supplemental Data Set 1 online was analyzed with the PhyML program (Guindon and Gascuel, 2003) at http://www.atgc-montpellier.fr/phyml/ using the substitution model HKY85, 0 invariable sites, four substitution rate categories, SPR and NNI tree improvement, and the approximate likelihood ratio test (Anisimova and Gascuel, 2006). The neighbor-joining tree (Saitou and Nei, 1987) of individual repeats (see Supplemental Data Set 2 online) was calculated at http://www.ebi.ac.uk/Tools/clustalw2/index.html (correct distance off and ignore gaps off).

Accession Numbers

Sequence data from *Arabidopsis* and *Chlamydomonas* MRL1 can be found in the Arabidopsis Genome Initiative (agi|At4g34830) or Joint Genome Institute databases (jgi|Chlre4|206534|OVA_OVA_Chlre2_kg.scaffold_1200023).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. DNA Gel Blot Analysis of the *MRL1* Locus.

Supplemental Figure 2. Alignment of MRL1 Protein Sequences.

Supplemental Figure 3. The *Chlamydomonas rbcL* mRNA Is a Primary Transcript.

Supplemental Figure 4. Phylogenetic Analysis of Individual PPRs from Plant and Algal MRL1.

Supplemental Figure 5. At-*mrl1* Does Not Affect Other Transcripts Than *rbcL*.

Supplemental Figure 6. Effect of At-*mrl1* on Rubisco Accumulation and *rbcL* Polysome Loading.

Supplemental Figure 7. Effect of High Light Treatment on PSI and PSII in the Wild Type and the *mrl1* Mutant.

Supplemental Figure 8. Alignment of Chloroplast Genome Sequences Upstream of *rbcL* Translation Start Site from Chlorophytes.

Supplemental Figure 9. Alignment of *rbcL* Promoter and 5′ UTRs from Streptophytes.

Supplemental Table 1. List of Primers Used.

Supplemental Data Set 1. Alignment (FASTA Format) of MRL1 Sequences Used to Construct Supplemental Figure 2 and the Cladogram Presented in Figure 3B.

Supplemental Data Set 2. Sequence of Pentatricopeptide Repeats Used in Supplemental Figure 4.

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4. Discussion

4.1 Binary set of factors for protein synthesis in the chloroplast of *Chlamydomonas*

Coordination of nuclear and chloroplast gene expression is mediated in several ways. Examples for post-transcriptional control include ribozymes / riboswitches. Ribozymes are catalytic RNAs that require little or no presence of accessory proteins. Riboswitches are RNA sensors that regulate post-transcriptional gene expression in response to temperature, other RNAs or metabolites (reviewed in Serganov and Patel 2007).

However, control usually is mediated by nuclear encoded regulatory factors acting on all levels of gene expression in the chloroplast. Several recurring themes for regulation by these factors can be observed: the dependence of an RNA molecule's lifespan and translational status on structural elements in its untranslated regions as well as prevalent binding of protein factors to these regions. These protein factors are often gene-specific and exhibit sharply divided functions. The first set of factors is composed of maturation factors necessary for stabilization and processing of their target messages, while the second set, represented by translation factors, is essentially required for translational activation of the processed transcript while interacting simultaneously with a factor from the first set.

The first example for such a set of factors is the Nac2/RBP40 complex which was investigated in more detail in the course of this thesis. This HMW complex participates in the early steps of D2 synthesis in *Chlamydomonas* by interacting with the *psbD* mRNA (Boudreau, *et al.* 2000, Schwarz, *et al.* 2007, Schwarz and Nickelsen 2010, Schwarz, *et al.* 2011). The *psbD* 5`UTR starts 74 nucleotides upstream of the start codon, but this full-length transcript is processed by degradation of the first 27 nucleotides independent of the presence of Nac2 (Nickelsen, *et al.* 1999). The processed region between the upstream positions -79 and -47 is recognized by an unidentified membrane-bound protein of approximately 47 kDa which eventually mediates Nac2 binding (Nickelsen, *et al.* 1994). The processed transcript requires the first twelve nucleotides as well as interaction of Nac2 with a region between positions -32 and -26 for stability (Nickelsen, *et al.* 1999). Necessary for efficient translation are two additional sequences in the *psbD* 5'UTR, one for binding of RBP40 and the other one possibly involved in ribosomal binding by its complementarity to 16S rRNA (Nickelsen, *et al.* 1999, Klinkert, *et al.* 2006). Therefore, the TPR protein Nac2 is the required

maturation/stabilization factor responsible for stabilizing the processed *psbD* message prior to its interaction with the translation factor RBP40 to initiate protein synthesis (Nickelsen, *et al.* 1999, Boudreau, *et al.* 2000, Ossenbühl and Nickelsen 2000). Nac2 seems to be integral for the formation of this complex as the whole complex appears to be disassembled in a *nac2* mutant (see figure 9 in 3.1: Schwarz, *et al.* 2007). This is in agreement with the proposed main role of TPR proteins as mediators of protein-protein interactions (D'Andrea and Regan 2003). Also fitting into this picture of a protein scaffold is that neither for Nac2 nor for Mbb1, another TPR protein involved in *psbB* mRNA stabilization, a direct binding to RNA could be reported (Boudreau, *et al.* 2000, Vaistij, *et al.* 2000b).

Structural models of RBP40 provided evidence that the repeats of this translational activator belong to the "heterogeneous nuclear ribonucleoprotein K homology (KH)" or "RNA binding domain (RBD)" families (Barnes, *et al.* 2004). KH domains were first identified in the human heterogeneous nuclear ribonucleoprotein K. Since then, proteins with KH motifs were found in other eukaryotes as well as in prokaryotes (reviewed in Adinolfi, *et al.* 1999). These RNA/ssDNA binding proteins contain several copies of one motif of ~ 70 amino acids and serve in processes like splicing or translational control. Intriguingly, their target recognition is unique. A single KH domain binds only four nucleotides and higher specificity is acquired by tandem arrangement of more than one domain or by proximity of other recognition motifs (reviewed in Valverde, *et al.* 2008). Another organellar protein with a KH motif is the polynucleotide phosphorylase participating in mRNA degradation in spinach chloroplasts (Yehudai-Resheff, *et al.* 2003). Also interesting are RBD proteins because there are examples that would fit into the possible modus operandi of RBP40. This includes helicases altering the secondary structure of dsRNA (Hernandez, *et al.* 2010). Alteration of a RNA structure could be the role of interaction between RBP40 and the sterically blocked *psbD* start codon during translation initiation. Evidence supporting this presumption is the abolished D2 protein synthesis in the strain in which the poly(U)-region of the *psbD* mRNA for RBP40 binding was replaced (Klinkert, *et al.* 2006). An additional/alternative role for RBP40 might be the enhancement of interaction between the *psbD* transcript and ribosomal RNA. This guidance function was reported for a RBD motif-containing helicase in organellar RNA editing (Seiwert and Stuart 1994, Hernandez, *et al.* 2010). Similar to RBP40 several mitochondrial RNA binding proteins, isolated from potato, were described to preferentially bind to poly(U)-rich sequences. Among those mitochondrial RNA-binding proteins was the chaperone HSP60 (Vermel, *et al.* 2002). Interestingly, the chloroplast isoform of the HSP60 chaperone system in *Chlamydomonas* has been reported to bind RNA, too (Balczun, *et al.* 2006). However, it is

conceivable that chaperones, during their involvement in regulation of protein folding, only co-migrate with RNA binding complexes (Goldschmidt-Clermont 2009). This is intriguing as RNA-containing complexes were also detected when members of the HSP70 chaperone system were investigated in *Chlamydomonas* chloroplasts in this thesis (see 3.5: Dorn, *et al.* 2010). Since these chaperones seem to fulfill a more general function, it is possible that they interact with HMW complexes involved in translation to regulate post-transcriptional steps of chloroplast gene expression.

Participation of maturation/stabilization factors in *Chlamydomonas* is not specific to the *psbD* message, as a secondary structure in the very beginning of the 5`UTR of the *rbcL* transcript is necessary for stabilization of the *rbcL* message. Binding of the PPR protein MRL1 to the first nucleotides of the *rbcL* 5'UTR is required for transcript accumulation, turning it into a prerequisite for RbcL protein synthesis (Suay, *et al.* 2005, see 3.6: Johnson, *et al.* 2010). Also similar to D2 synthesis is the expression of *petA,* encoding the cytochrome f subunit of the cytochrome b_6f complex. MCA1, the first described member of the PPR protein family in *Chlamydomonas*, functions as a stabilization factor of the *petA* transcript, whose start codon is part of a complex secondary RNA structure, similar to the situation in the *psbD* and *rbcL* transcripts (Loiselay, *et al.* 2008). The amount of *petA* transcripts is under control of MCA1 whereas the protein level is limited by the availability of the translational regulator TCA1 (Raynaud, *et al.* 2007). These factors form multimers by interacting with each other, possibly already accounting themselves for the observed size of the described HMW complex (Boulouis, *et al.* 2011). Also in higher plants a similar mechanism of modulating the stability and translation by binding of the protein PPR10 to the *atpH* transcript has been proposed for efficient translation of the respective mRNA (Prikryl, *et al.* 2011).

Interaction of regulatory factors with target RNAs is not always limited to the untranslated regions. MDA1 in *Chlamydomonas* mediates its stabilizing function by interacting with the ORF of the *atpA* transcript (Drapier, *et al.* 2002). An additional example for regulation through an ORF is the interaction between the *psaC* coding region and the *ndhD* 5`UTR in tobacco chloroplasts. This represses translation of both the *psaC* and the *ndhD* transcripts (Hirose and Sugiura 1997).

Further experiments have shown that several previously uncharacterized proteins are involved in post-transcriptional regulation of chloroplast gene expression in different organisms. The precise modus operandi of the investigated proteins and/or their participation in HMW complexes was not always elucidated (examples include Stampacchia, *et al.* 1997, Zerges, *et*

al. 1997, Bunse, *et al.* 2001, Auchincloss, *et al.* 2002, Dauvillée, *et al.* 2003, Nakamura, *et al.* 2003, Sane, *et al.* 2005, Barneche, *et al.* 2006, Schult, *et al.* 2007, Müller, *et al.* 2011). Therefore, the assumption of the presence of complementing sets of maturation/stabilization and translation factors as it was found for *psbD* and *petA* transcripts is tempting. Promising candidates are represented by transcripts when one of the involved regulatory proteins is already known (see section 1.4.2, table 1; section 1.4.3, table 2).

Thus, available data suggest the presence of a binary set of transcript-specific factors which might be associated with additional and less specific proteins in high molecular weight complexes - resulting from size and number of involved factors and transcripts - to control post-transcriptional steps of gene expression in the chloroplast (examples include Boudreau, *et al.* 2000, Vaistij, *et al.* 2000b, see 3.1: Schwarz, *et al.* 2007, see 3.6: Johnson, *et al.* 2010, Boulouis, *et al.* 2011, see 3.3: Schwarz, *et al.* 2011). A generalized scheme of this process is depicted in figure 5.

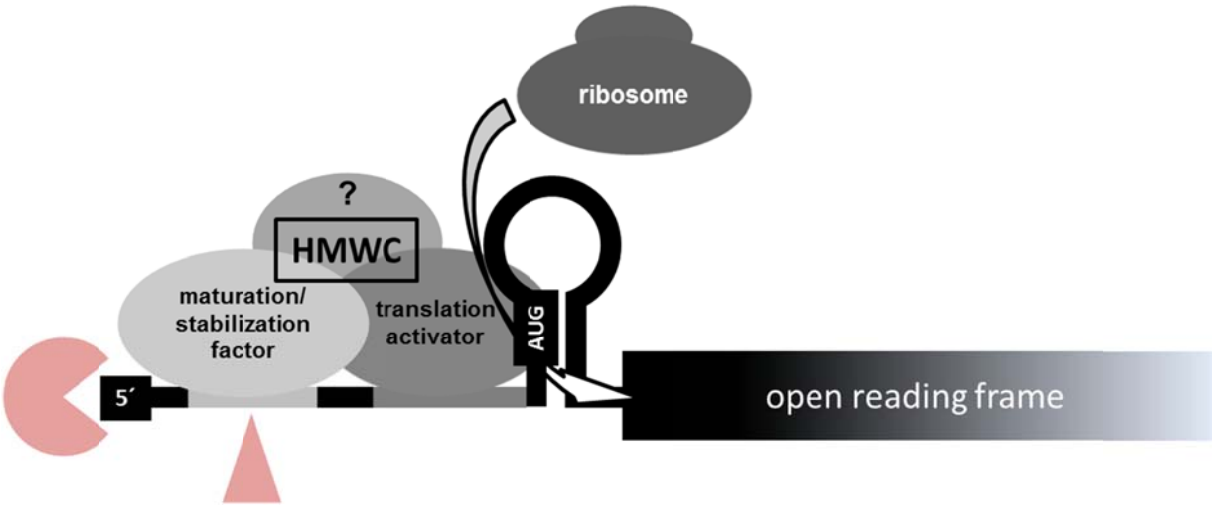

Figure 5: Model for a binary system of maturation and translation factors at the 5'UTR of plastidial transcripts. Binding of maturation/stabilization factors to the 5'UTR mediates transcript processing and/or stabilization by protecting the RNA from degradation by generic and/or specific exo/endonucleases (depicted in orange). Binding of a translation activator, which might be enhanced by interaction with the already bound maturation/stabilization factor, potentially alters the secondary structure of the RNA around the AUG start codon. Additional components possibly bind and regulate complex formation depending on organellar conditions. The resulting HMW complexes (HMWC) control access of ribosomes to the open reading frame for efficient protein synthesis in accordance with requirements of the cell. Interaction/binding between factors and RNA sequences is indicated by similar colors.

4.2 Influence of post-translational changes on protein synthesis

Based on the assumption that chloroplast translation is controlled by a system of several factors interacting with a target message, research also focuses on the identification of mechanisms regulating these interactions. Even though regulation of plastid gene expression occurs at several levels (see I.4), activity of mature proteins can also be influenced by modifications at the post-translational level in response to external signals. These signals include in particular light quantity and quality, which might lead to changes especially in phosphorylation as described for the PSII core proteins D1, D2, CP43, CP47 and PsbH (reviewed in Pesaresi, *et al.* 2011). Another significant post-translational modification (PTM) that regulates protein activity is the glutathionylation of proteins via their cysteine residues. Thioredoxin f is an important example in the redox signaling pathways of the chloroplast (reviewed in Lemaire, *et al.* 2007, Montrichard, *et al.* 2009). This field of work is supported by proteomic approaches detecting specific modifications of chloroplast proteins as a function of changing external signals, i.e. the compilation of maps of chloroplast sub-fractions (Yamaguchi, *et al.* 2003, Förster, *et al.* 2006).

In case of RBP40, one of these analyses in *C. reinhardtii* which was based on a mass-spectrometrical identification of isoelectrically focused stromal proteins did not reveal N-terminal acetylation to be a PTM of RBP40 as it is reported for D1, D2 and RbcL (Bienvenut, *et al.* 2011). Instead, Michelet and co-workers found RBP40 to be a target for glutathionylation under induced oxidative stress, which always occurs at the thiol group of cysteine residues (2008). The only cysteine residue within the RBP40 protein sequence is located in the proposed transit peptide (Barnes, *et al.* 2004). Subsequently, there must be an unusually short transit peptide upstream of that cysteine (Schwenkert, *et al.* 2011). RBP40 could also localize to the stroma by an alternative import pathway, which does not involve processing of the import signal, as the size of full-length RBP40 did not change after *in vitro* chloroplast import experiments (Barnes, *et al.* 2004). The occurrence of a cysteine residue in the mature RBP40 protein is further supported by a cysteine-specific thiol-alkylation of the native RBP40 protein shown in this thesis (see 3.3: Schwarz, *et al.* 2011). Thus, RBP40 is likely regulated by redox-dependent glutathionylation of its single cysteine residue, which is a regulator of its association to the Nac2 complex and therefore the initiation of D2 synthesis (see 3.3: Schwarz, *et al.* 2011). Additionally, association of RBP40 could be affected by the redox state of its interaction partner Nac2, a protein containing eleven cysteine residues. Interestingly, the last two of those are located within the C-terminal TPR domains of Nac2,

whose redox state might influence the protein structure of that TPR protein to change the ability of Nac2 for protein-protein interaction or RNA-binding capacity (see 3.3: Schwarz, *et al.* 2011).

Another mechanism to post-translationally regulate the activity of proteins involved in translation is represented by methylation of protein arginines. This modification mainly targets RNA-binding proteins and has been shown in mammalian cells (Pahlich, *et al.* 2006, Bedford 2007, Blackwell, *et al.* 2010). So far, there is no experimental proof that also RBP40 is regulated by arginine methylation. However, *in silico* analysis revealed a target motif for an arginine-specific methyltransferase, even though until now, no such transferases have been characterized in chloroplasts. *In silico* analyses of annotated and predicted *Chlamydomonas* genes show that there are transferase homologues with putative chloroplast localization (Schwarz and Nickelsen, unpublished results). In combination with a potential target motif in RBP40, a comparable additional regulation of D2 synthesis by methylation of RBP40 is a tempting speculation. Methylation of arginine residues was recently shown to decrease the RNA-binding activity of the cytosolic translation repressor NAB1. Oxidative modifications (including redox-dependent glutathionylation) of the cysteine residues in NAB1's RNA recognition motif (RRM) also have a negative influence on the protein's RNA binding capacity (see 3.4: Wobbe, *et al.* 2009, Blifernez, *et al.* 2011).

The requirement of binding ATP-binding for HSP70B to enable interactions with CDJ3 is another example for potentially PTM-induced conformational changes as CDJ3 is present in RNA-containing complexes and its redox-reactive ferredoxin domains could regulate chaperone interaction with specific substrates (see 3.5: Dorn, *et al.* 2010). Taken together, results achieved during this thesis provide interesting insights into mechanisms regulating plastidic translation by post-translational modification of involved proteins.

4.3 Influence of the organellar redox state on chloroplast translation activity

Change of translation rates by redox levels as described for the translational repressor NAB1 does not only take place in the cytosol. Reversible formation of a disulfide bridge also influences *psbD* translation in the chloroplast of *C. reinhardtii* as it was shown in this thesis. Establishment of that disulfide bridge associates RBP40 to Nac2 for efficient D2 synthesis in the light (see 3.3: Schwarz, *et al.* 2011). The assembly of translational activators to a target

RNA is an important key player of organellar gene expression as translation is supposed to be the pacemaker of protein synthesis in the chloroplasts (Eberhard, *et al.* 2002). In a comparable manner redox-dependent interaction of several proteins involved in D1 synthesis was proposed (see 1.4.3, Barnes and Mayfield 2003, Somanchi, *et al.* 2005, Alergand, *et al.* 2006). This also holds true for the light dependence of the synthesis rates of the D1 and D2 proteins (Malnoë, *et al.* 1988, Trebitsh, *et al.* 2000, see 3.3: Schwarz, *et al.* 2011).

The redox regulation of the synthesis of the D2 protein presents a certain challenge. The disulfide bridge that is necessary for assembly of the Nac2/RBP40 complex and subsequent translation of *psbD* mRNA is formed in the light and opened in the dark. Lack of photosynthetic electron flow during the night cannot reduce this disulfide bridge, whereas during the day this covalent bond exists in presence of reducing equivalents that are produced from photosynthesis. It is shown in this thesis that a possible source in *Chlamydomonas*, supplying the required electrons for reduction of the Nac2-RBP40 disulfide bridge in the dark, could be the NADPH-dependent thioredoxin reductase class C (NTRC) system (see 3.3: Schwarz, *et al.* 2011), which is exclusive to photosynthetic organisms (Pascual, *et al.* 2011). In comparison to other NADPH-dependent thioredoxin reductases, NTRC contains an additional thioredoxin domain and offers an alternative electron pathway independent of light and ferredoxin. The thioredoxin is reduced by electrons from NADPH by using FAD as a co-factor (Chibani, *et al.* 2010). This alternative electron source is possible in the dark as NADPH can be generated by the oxidative pentose phosphate pathway (Neuhaus and Emes 2000). One of the described functions of this enzyme was the reduction of thiol-dependent peroxidases during stress responses in *Arabidopsis* (Moon, *et al.* 2006). NTRC mutants were hypersensitive to extended periods of darkness due to accumulation of hydrogen peroxide whereas electrons - required for peroxide reduction - can be provided by ferredoxin during the day (Pérez-Ruiz, *et al.* 2006). Other pathways affected by NTRC include functions during biogenesis of aromatic compounds and chlorophyll as well as protection against abiotic stress (Serrato, *et al.* 2004, Stenbaek, *et al.* 2008, Lepistö, *et al.* 2009). Moreover, it has been linked to the regulation of starch synthesis in combination with the ferredoxin/thioredoxin system (Ballicora, *et al.* 2000, Michalska, *et al.* 2009). Data provided here suggest an additional role of this multifunctional enzyme: the involvement in chloroplast gene expression in the dark.

The finding of a possible regulatory function of NTRC in synthesis/assembly of PSII reinforces the linkage between intracellular transport, metabolism and nutrient availability by alteration of redox states and post-translational modifications (Geigenberger, *et al.* 2005,

Bräutigam, *et al.* 2009, see 3.4: Wobbe, *et al.* 2009, Balsera, *et al.* 2010, Dorn, *et al.* 2010, Blifernez, *et al.* 2011, see 3.3: Schwarz, *et al.* 2011). In conclusion, a further validation of the involvement of the NTRC system in D2 synthesis would provide a direct link between expression of chloroplast genes and carbon metabolism in *C. reinhardtii*. This would provide an important crosstalk between photosynthesis and catabolic cell activities. Further details that await elucidation include the identification of the cysteine residue of Nac2 interacting with RBP40, the elucidation of the function of a predicted NADPH binding site in Nac2 and the characterization of the electron acceptor necessary for the formation of the disulfide bridge between Nac2 and RBP40.

A further example of a chloroplast protein whose translation relies on the organellar redox state is the large subunit of Rubisco. Synthesis of that protein responds to shifts in the glutathione pool caused by oxidative stress (Irihimovitch and Shapira 2000). Oxidative events at thiol groups in the Rubisco protein induce a conformational change which leads to the exposure of an N-terminal structure with homologies to RRM domains and the human U1A splicing factor. This change of protein confirmation, possibly assisted by chloroplast chaperones of the HSP60 family, enables Rubisco to bind RNA unspecifically under oxidizing conditions (Hemmingsen, *et al.* 1988, Yosef, *et al.* 2004). Binding of the protein to RNAs in its vicinity, including *rbcL* mRNA, might stall protein synthesis to lessen the effects of oxidative stress. (Cohen, *et al.* 2005). Further results also showed a co-regulation of chaperones belonging to HSP70 complexes and stress conditions as well as their involvement in the synthesis of bacterial Rubisco (Checa and Viale 1997, Shrager, *et al.* 2003).

Additionally, HSP70 was found to be a potential thioredoxin target itself (Lemaire, *et al.* 2004). During this thesis, it was shown for members of the HSP70 chaperone family in *Chlamydomonas* that chaperone complexes themselves contain RNA. It was not elucidated so far if the interaction with RNA is direct or if the chaperone binds co/post-translationally to the protein encoded by that RNA (see 3.5: Dorn, *et al.* 2010). This gives rise to the opportunity that the organellar redox state directly regulates translational activity for plastidial proteins. As an alternative, translational regulation could occur via feedback mechanisms through disassembly of resulting protein complexes in response to the oxidation level of a cellular compartment. An example of translational regulation by the assembly state of the resulting complex is the synthesis of PSII core subunits in *Chlamydomonas* according to the CES principle, e. g. *psbB* translation also depends on redox-dependent D2 synthesis in addition to

the level of available chlorophyll (see 1.4.3, Eichacker, *et al.* 1992, Plumley and Schmidt 1995, Minai, *et al.* 2006).

These examples of post-transcriptional regulatory events for synthesis of proteins show that fluctuating interactions between several factors are necessary for maintaining cellular survival while adapting to external (e.g. light conditions) and internal aspects (e.g. available interaction partners).

In conclusion, the results of this thesis enhanced the understanding of several aspects that fulfill important functions in the post-transcriptional regulation of chloroplast gene expression in *C. reinhardtii*.

5. Appendix

5.1 List of publications

During the progress of this thesis the following papers were published or are in submission.

Research articles

Schwarz, C., Bohne, A.V., Cejudo, F.J. and Nickelsen, J. (2011) An intermolecular disulfide-based light switch for chloroplast *psbD* gene expression in *Chlamydomonas reinhardtii*. *Plant J.*, (submitted)

Dorn, K.V., Willmund, F., Schwarz, C., Henselmann, C., Pohl, T., Hess, B., Veyel, D., Usadel, B., Friedrich, T., Nickelsen, J. and Schroda, M. (2010) Chloroplast DnaJ-like proteins 3 and 4 (CDJ3/4) from *Chlamydomonas reinhardtii* contain redox-reactive Fe-S clusters and interact with stromal HSP70B. *Biochem. J.*, **427**, 205 – 215

Johnson, X., Wostrikoff, K., Finazzi, G., Kuras, R., Schwarz, C., Bujaldon, S., Nickelsen, J., Stern, D.B., Wollman, F.A. and Vallon, O. (2010) MRL1, a conserved pentatricopeptide repeat protein, is required for stabilization of *rbcL* mRNA in *Chlamydomonas* and *Arabidopsis*. *Plant Cell*, **22**, 234 – 248

Schwarz, C. and Nickelsen, J. (2010) Enrichment of native, high molecular weight ribonucleoprotein complexes from chloroplast by consecutive gel filtration steps. *Endocyt. Cell Res.*, **20**, 89 – 94

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

Schwarz, C., Elles, I., Kortmann, J., Piotrowski, M. and Nickelsen, J. (2007) Synthesis of the D2 protein of PSII in *Chlamydomonas* is controlled by a high molecular mass complex containing the RNA stabilization factor Nac2 and the translational activator RBP40. *Plant Cell*, **19**, 3627 – 3639

Reviews

Bohne, A.V., Schwarz, C., Jalal, A., Ossenbühl, F. and Nickelsen, J. (2009) Control of organellar gene expression in *Chlamydomonas reinhardtii* – future perspectives. *Endocyt. Cell Res.*, **19**, 70 – 80

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Ehrenwörtliche Versicherung

Ich versichere hiermit ehrenwörtlich, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Beihilfen angefertigt wurde.

Dachau, den 16. Januar 2012 Christian Schwarz

Erklärung

Hiermit erkläre ich, dass diese Dissertation weder ganz noch in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt wurde. Weiterhin habe ich mich nicht anderweitig einer Doktorprüfung ohne Erfolg unterzogen.

Dachau, den 16. Januar 2012 Christian Schwarz