Molecular and functional analysis of photosynthesis-related mutants from *Chlamydomonas reinhardtii* and *Arabidopsis thaliana*

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ABSTRACT

Synthesis and assembly of plastidial proteins and protein complexes is mainly regulated by nucleus-encoded factors which act on various steps of gene expression. The present study was aimed to identifying such regulatory factors involved in the expression and assembly of photosystem II subunits by a forward genetic approach.

16 nuclear insertion mutants from the green alga *C. reinhardtii* exhibiting a PSII deficient phenotype were used to identify potentially new candidate proteins. Following an initial phenotypical characterization, including the analysis of chloroplast transcripts, protein synthesis and accumulation, eight mutants were selected for the identification the underlying genetic cause of a mutant phenotype. By hybridizations of genomic DNA, as well as PCR or map based approaches for five out of these eight mutants the corresponding mutated gene could be identified. Only one mutant was verified to possess a mutation which is allelic to a previously described one, *nac2*, involved in *psbD* mRNA stabilization. However, four genes were identified encoding for proteins not known so far to be involved in PSII synthesis: a putative transcription factor (mutant *45a*), a speract/scavenger receptor domain protein (mutant *42b*), OHP2 (one helix protein 2, mutant *101a*), a member of the light-harvesting-like (LIL) protein family, as well as CLR24 (mutant *101b*), a member of the OPR (octotricopeptide repeat) repeat protein family.

To elucidate the role of OHP2 and CLR24 in photosynthesis, a detailed molecular and phenotypical characterization of the mutants was performed. At this, a specific function of OHP2 in accumulation of the PSII reaction center protein D1 was observed. Pulse labeling and transcript hybridization experiments suggested a role of OHP2 either in the translation process of the *psbA* mRNA or in stabilization of the encoded D1 protein.

The second protein, CLR24, belongs to the OPR family, whose members are thought to fulfill diverse functions during post-transcriptional regulation in chloroplasts via predicted RNA binding capacities. A biochemical analysis showed a function of CLR24 in the formation of PSII dimers and super complexes, but not PSII monomers. Furthermore, transcript hybridizations of the *clr24* mutant revealed an altered processing of polycistronic *atpA-psbI-cemA-atpH* messages, which leads to the loss of mature *psbI* transcripts, encoding a small PSII subunit described to be involved in PSII dimer stabilization. Therefore, a role of CLR24 in stabilization/processing of the *psbI* transcript is indicated, whose absence causes a defect in PSII complex formation and reduced photosynthetic activity.

To investigate the function of the single OPR protein identified in *Arabidopsis thaliana*, AtRAP, T-DNA insertion lines were subjected to phenotypical and biochemical analyses. *AtRAP* mutants revealed growth retardation, a pale green phenotype, and reduced photosynthetic activity. Furthermore, the mutants exhibited normal levels of abundant chloroplast transcripts, whereas their translation and therefore accumulation of chloroplast encoded proteins was dramatically reduced in early growth stages. RNA hybridizations showed a severely affected maturation of 16*S* rRNA: while decreased levels of mature 16*S* rRNA were detected in *AtRAP* T-DNA lines, a larger precursor accumulated as compared to the wild-type. Therefore, a function of AtRAP in 16*S* rRNA processing is postulated.

ZUSAMMENFASSUNG

Die Synthese und Assemblierung plastidärer Proteine und Proteinkomplexe wird vor allem durch kernkodierte Proteine reguliert, die auf verschiedenen Ebenen der Genexpression wirken. Ziel der vorliegenden Arbeit war eine Identifizierung solcher, in die Expression und Assemblierung von Untereinheiten des Photosystems II involvierter Faktoren, durch einen vorwärts gerichteten genetischen Ansatz.

Zur Identifizierung neuer potentieller Kandidatenproteine wurden 16 Insertionsmutanten der Grünalge *Chlamydomonas reinhardtii* verwendet, die einen PSII-defizienten Phänotyp aufwiesen. Nach einer initialen phänotypischen Charakterisierung, die die Analyse plastidärer Transkripte, der Proteinsynthese- und akkumulation umfasste, wurden acht Mutanten zur Identifizierung der zugrunde liegenden genetischen Ursache des Mutantenphänotyps, ausgewählt. Mit Hilfe von Hybridisierungen genomischer DNA, sowie Karten- und PCR-basierten Ansätzen war es möglich, für fünf der acht Mutanten das korrespondierende mutierte Gen zu identifizieren. Hierbei wurde lediglich für eine der Mutanten eine Mutation verifiziert, die allelisch zu einer bereits zuvor beschriebenen im *Nac2*-Lokus ist, der eine Rolle in der *psbD* mRNA Stabilisierung spielt. Dahingegen wurden vier Gene identifiziert, die für Proteine kodieren, von denen eine Involvierung in die PSII-Synthese bislang unbekannt war: ein putativer Transkriptionsfaktor (Mutante *45a*), ein *speract/scavenger* Rezeptordomänen-Protein (Mutante *42b*), OHP2 (*one helix protein* 2, Mutante *101a*), ein Vertreter der LIL (*light-harvesting-like*) Proteinfamilie, sowie CLR24, einen Vertreter der OPR (octotricopeptide repeat) Proteinfamilie (mutant *101b*).

Zur Aufklärung der Rolle von OHP2 und CLR24 in der Photosynthese wurde eine detaillierte molekulare und phänotypische Charakterisierung der Mutanten vorgenommen. Hierbei konnte eine spezifische Funktion von OHP2 in der Akkumulation des PSII Reaktionszentrumproteins D1 beobachtet werden. Pulsmarkierungs- und Transkripthybridisierungsexperimente suggerieren hierbei entweder eine Rolle von OHP2 im Translationsprozess der *psbA* mRNA oder aber in der Stabilisierung des kodierten D1 Proteins.

Das zweite Protein, CLR24, gehört zur OPR Familie, von deren Vertretern angenommen wird, dass sie mit Hilfe einer vorhergesagten RNA-Bindungsfähigkeit diverse Funktionen während der post-transkriptionellen Regulation in den Chloroplasten erfüllen. Eine biochemische Analyse zeigte hierbei, dass CLR24 in die Formation von PSII-Dimeren und –Superkomplexen, nicht aber die von PSII-Monomeren involviert ist. Des Weiteren zeigten Transkripthybridisierungen der *clr24* Mutante eine veränderte Prozesszierung des

polycistronischen *atpA-psbI-cemA-atpH* Transkriptes, die zum Verlust reifer *psbI* mRNA führt. Die *psbI* mRNA kodiert eine kleine Untereinheit des PSII, die eine beschriebene Funktion in der PSII-Dimerformation aufweist. Es wird daher eine Rolle von CLR24 in der Stabilisierung/Prozessierung des *psbI* Transkriptes angenommen, dessen Abwesenheit einen Defekt der PSII Komplexformation und reduzierte photosynthetische Aktivität mit sich bringt. Um die Funktion des einzigen in *Arabidopsis thaliana* identifizierten OPR Proteins, AtRAP, zu untersuchen, wurden entsprechende T-DNA Insertionslinien einer phänotypischen und biochemischen Analyse unterzogen. Die *AtRAP*-Mutanten zeigten hierbei ein verzögertes Wachstum, einen hellgrünen Phänotyp, sowie reduzierte photosynthetische Aktivität. Des Weiteren wiesen die Mutanten normale Mengen abundanter plastidärer Transkripte auf, wohingegen die Translation und die damit verbundene Akkumulation Chloroplasten-kodierter Proteine in frühen Wachstumsstadien dramatisch reduziert waren. RNA-Hybridisierungen zeigten einen deutlichen Effekt auf die Reifung der 16*S* rRNA: während verringerte Mengen reifer 16*S* rRNA detektiert wurden, akkumulierte im Vergleich zum Wildtyp ein längerer Vorläufer in den *AtRAP* T-DNA Linien. Es wird daher eine Funktion des AtRAP Proteins in der 16*S* rRNA Prozessierung postuliert.

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ABBREVIATIONS

APS	Ammonium persulfate
A. thaliana	*Arabidopsis thaliana*
ATP	Adenosine triphosphate
BLAST	Basic alignment search tool
℃	Degree Celsius
C. reinhardtii	*Chlamydomonas reinhardtii*
cDNA	Complementary deoxyribonucleic acid
Chl	Chlorophyll
Ci	Curie
CO_2	Carbon dioxide
cTP	Chloroplast transit peptide
Da	Dalton
ddH_2O	Double destilled water
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylene diamin tetraacetic acid
g	Force of gravity
gDNA	Genomic deoxyribonucleic acid
H_2O_2	Hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMW	High molecular weight
kb	Kilobase(s)
knt	Kilonucleotide(s)
L	Litre
LEF	Linear electron flow
LHC	Light harvesting complex
LMW	Low molecular weight
M	Mole(s) per litre
min	Minute
MCS	Multiple cloning site
mRNA	Messenger RNA
$MgCl_2$	Magnesium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NDH	NAD(P)H dehydrogenase complex
NEP	Nuclear encoded (plastidial) RNA-Polymerase
nt	Nucleotide(s)
(d)NTP	(Deoxy) nuclesidetriphosphate
OD	Optical Density
OPR	Octotricopeptide repeat

ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PC	Plastocyanin
PEP	Plastid encoded (plastidial) RNA-Polymerase
pH	Negative decimal logarithm of proton activity
p*I*	Iso-electric point
PPR	Pentatricopeptide repeat
PQ	Plastoquinone
PSI	Photosystem I
PSII	Photosystem II
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
RNase	Ribonuclease
RNAP	RNA polymerases
rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
rRNA	Ribosomal RNA
RuBisCo	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
TPR	Tetratricopeptide repeat
Tris	Tris(hydroxymethyl)-aminomethane
tRNA	Transfer RNA
U	Units
UTR	Untranslated region
UV	Ultra violet
v/v	Volume per volume
w/v	Weight per volume
WT	wild-type
β-DM	ß-dodecylmaltoside
µ	Micro

1 INTRODUCTION

Photosynthesis gives plants, algae, and cyanobacteria the ability to use sunlight to extract electrons from water, at this providing energy for growth. In plants and algae, photosynthesis is performed in chloroplasts.

1.1 Endosymbiosis and chloroplast gene transfer

It is widely accepted that chloroplasts, similar to mitochondria, descended from a free-living bacterial ancestor, which invaded or was engulfed by a mitochondrion-possessing eukaryote between 1.5 and 1.2 billion years ago. Due to these endosymbiotic events, ongoing gene transfer events from organelle to nucleus are observed in eukaryotic photosynthetic organisms, leading to severely reduced organellar genomes (reviewed in Kutschera and Niklas, 2005). Nowadays only a few proteins (~100) are encoded in the chloroplast genome, among which are proteins for transcription (RNA polymerase subunits), translation (ribosomal proteins, rRNAs and tRNAs), as well as photosynthesis (Sato et al., 1999). Approximately 4500 proteins of the ancestral endosymbiont are currently encoded in the nucleus (Martin et al., 2002; Timmis et al., 2004). For instance, the chloroplast genome of *A. thaliana* only contains 85 protein-encoding genes and 44 genes for structural RNAs (Sato et al., 1999). The rest of the chloroplastic proteins are encoded by the nuclear genome (reviewed in Jarvis and Soll, 2001). That means, most proteins (93% ~99%) found in organelles are encoded in the nucleus, synthesized in the cytoplasm and then imported into the organelles via N-terminal transit peptides.

The interdependence of genetic systems of chloroplasts, mitochondria and the nucleus requires an inter-compartmental signaling to allow for a coordinated interplay of the three compartments (Herrmann and Neupert, 2003).

1.2 Photosynthesis

Photosynthetic organisms, such as plants, green algae (eukaryotes) and cyanobacteria (prokaryotes) are defined as photoautotrophs due to their usage of sunlight to synthesize organic sugars from inorganic substances.

During photosynthesis, light energy is transformed into chemical energy in form of NADPH and ATP (light-dependent reactions), which are later employed by the light-independent Calvin-Benson cycle via the RuBisCo (Ribulose-1, 5-bisphosphate carboxylase oxygenase)

complex, to incorporate atmospheric carbon into organic compounds (Figure 1.1). The photosynthesis light reactions of eukaryotes take place in the chloroplast thylakoid membranes, and plasma membranes of prokaryotes. The cooperative actions of photosynthesis rely on four large protein complexes, i. e. the photosystems I and II (PSI and PSII), the cytochrome b_6f complex (Cyt b_6f) and an ATP synthase, and peripheral light-harvesting complexes (LHCs) which are together participating in the linear electron transport. Firstly, in LHCs, the photon-excited chlorophyll pigments (Chl*), either quench to the ground state via emitting fluorescence, or drive photochemical reactions by transferring energy to the PSII reaction center. The transferred energy is subsequently used to split H_2O into oxygen, protons, and electrons by the Oxygen Evolving Complex (OEC) attached to PSII. Later on, protons accumulating in the lumen generate a proton gradient across the thylakoid membrane, which can be used by the ATP synthase to produce ATP. Electrons transferred from PSII to PSI via the Cyt b_6f complex finally reduce $NADP^+$ to NADPH.

Each of the above four complexes contains multiple subunits encoded by both nucleus and chloroplast (Figure 1.1). For instance in higher plants, PSII comprises 27~28 subunits, Cyt b_6f 8 subunits, PSI 21 subunits, and the ATP synthase 9 subunits (Dekker and Boekema, 2005; Lennartz et al., 2001; McCarty et al., 2000; Zolla et al., 2007).

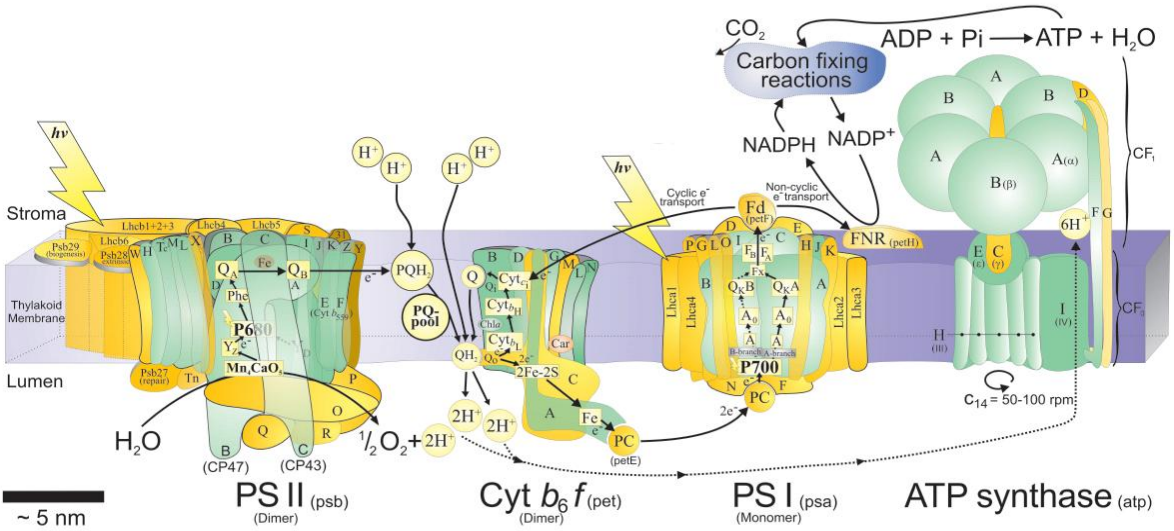


Figure 1.1 Major thylakoid proteins and protein complexes of the *Arabidopsis thaliana* chloroplast (adapted from Allen et al., 2011). Photosystem II (PSII), cytochrome b_6f (Cyt b_6f), photosystem I (PSI) and ATP synthase are shown. Polypeptide subunits encoded in the chloroplast are colored green; polypeptide subunits encoded in the nucleus are colored yellow. For further explanation, see text.

1.2.1 Photosystem II

As PSII confers a charge separation which results in water splitting and the production of O_2, it is considered as the key protein complex of photosynthesis light reactions. To elucidate the exact working mode of this protein-pigment super-complex, it is studied comprehensively in prokaryotes and eukaryotes. At this, researchers currently mainly focus on high-resolution structures of intact PSII complexes and its subunits, the interaction of these complexes into higher order organizations, as well as the identification of accessory protein factors involved in these assembly processes (reviewed in Kouril et al., 2012; Nixon et al., 2010).

1.2.1.1 The composition of photosystem II

Crystal structure data of PSII from various photosynthetic bacteria demonstrated that PSII complexes arrange to super-complexes with almost 1100 kDa (Dekker and Boekema, 2005). These super-complexes are composed of PSII dimers and light harvesting complexes (LHCs) possessing most of the sunlight-absorbing pigments. The monomeric PSII consists of many known subunits, the number of which is continuously increasing due to the usage of more sensitive electron microscopy (Allen et al., 2011). So far, almost 40 protein subunits have been revealed, among which the attachment sites of abundant subunits were clarified in cyanobacteria these years (Figure 1.2). D1 and D2 are located in the middle of the complex forming the reaction center (RC). Each of these proteins contains five transmembrane α-helices, which bind pigment-co-factors, like chlorophyll, pheophytin, and plastoquinone (reviewed in Schlodder et al., 2008; Sugiura et al., 2008).

CP43 and CP47, composing the core antenna, are located on either side of the RC, each possessing six transmembrane α-helices, which bind chlorophyll *a* and *β*-carotene. Additionally, Ferreira (2004) reported that CP43, together with D1, participates in the ligation of the $CaMn_4$ cluster, which is essential for water-splitting.

Moreover, a number of low molecular weight (LMW) proteins are surrounding these subunits, on the periphery of the complex, which are variable from cyanobacteria to chloroplasts depending on the species (reviewed in Enami et al., 2008 section 1.3.3.2) .As described above, the pigment binding LHCs also associate with PSII dimers as organism-dependent antenna systems: for instance, water-soluble, extrinsic phycobilisomes in cyanobacteria and red algae, and membrane-embedded light-harvesting chlorophyll-*a*/*b*-binding (CAB) subunits in chloroplasts (reviewed in Green, 2005).

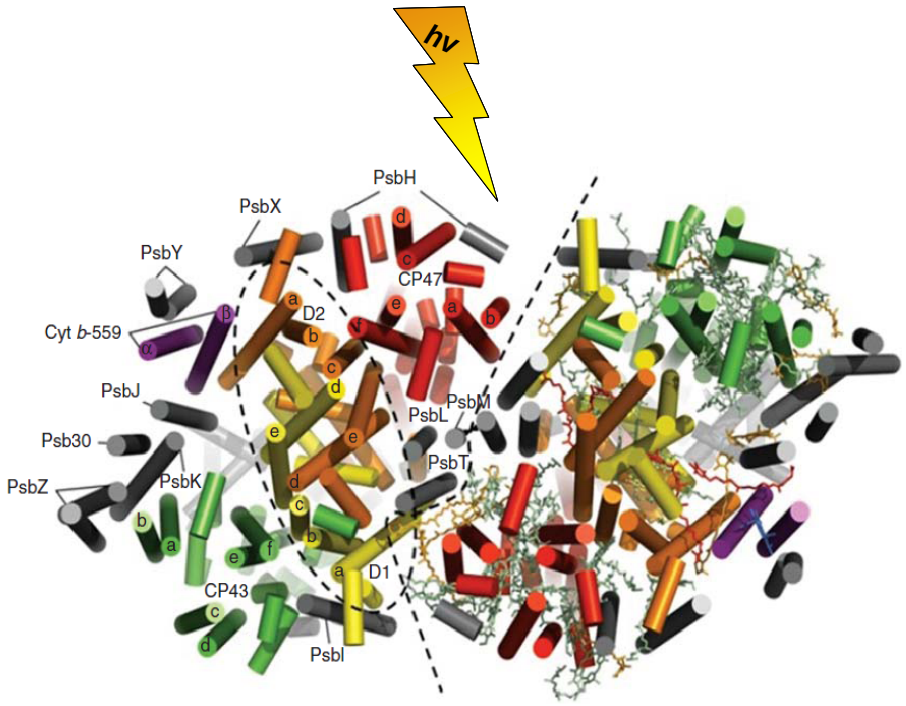


Figure 1.2 Subunit organization of a PSII dimer from cyanobacteria, viewed from the cytoplasmic side of the membrane (adapted from Nixon et al., 2010). Two PSII monomers are shown in the picture, separated by a black dashed line. The subunits are given in different colors in the monomer on the left side, such as D1 (yellow), D2 (orange), CP43 (green), CP47 (red), cytochrome b-559 (purple) and the remaining 11 small subunits (grey). The cylinders represent the α-helical elements of each subunit. The elliptical black dashed circle represents the D1–D2–Cyt b-559 sub-complex. The monomer on the right side is indicated with the same color coding system and represents the co-factors of PSII: chlorophylls (green), carotenoids (orange), pheophytins (yellow), plastoquinones (red), and haem (blue), shown in stick form.

1.2.1.2 The assembly of photosystem II

Even though the biogenesis of PSII complexes is also studied in green algae and higher plants, most detailed information originate from photosynthetic bacteria. A recent review from Nixon (2010) summarizes the PSII assembly process in *Synechocystis* sp. PCC 6803, which is shown in Figure 1.3. The assembly starts firstly from insertion of the anchor protein D2, which acts as a scaffold for subsequent steps, followed by multiple assembly steps which involve the participation of distinct protein factors, only some of which are found in the final functional PSII complexes (compare sections 1.2.1.3). Described in brief, the formation of the D2-Cyt*b*-559 sub-complex initiates the assembly of PSII monomers, and then a PSII RC-like complex is formed after the insertion of D1 and other small proteins into the D2-Cyt*b*559 sub-complex. Afterwards the RC47 complex is formed by insertion of CP47 into the PSII RC-like complex, followed by attachment of CP43 to form the monomeric PSII core complex (RCC1). This PSII core complex is the starting formation for light-driven assembly of the oxygen-evolving complex (OEC), which completes the formation of PSII monomers.

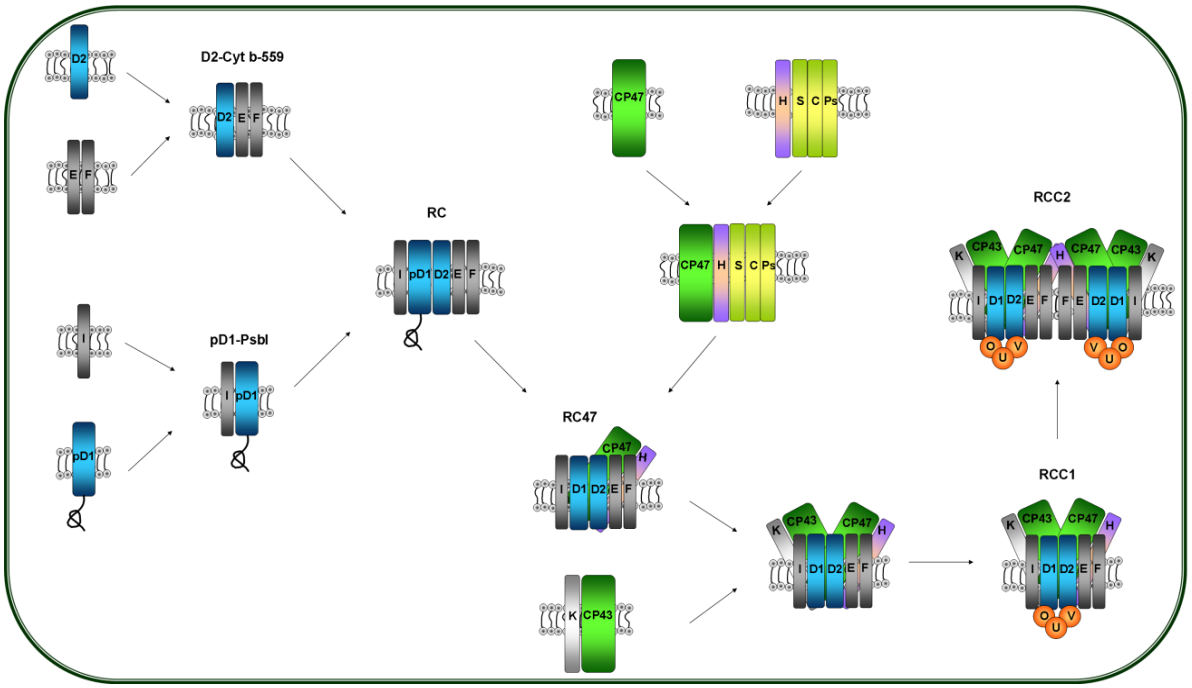


Figure 1.3 Assembly of the PSII complex in *Synechocystis* sp. PCC 6803 (adapted from Nixon et al., 2010). The upper case letters represent the corresponding LMW proteins: PsbE, PsbF, PsbH, PsbI and PsbK, as well as the extrinsic subunits PsbO, PsbU and PsbV. The small CAB-like proteins are indicated by small chlorophyll *a*/*b*-binding-like proteins (SCPs).

As mentioned above, PSII exists mainly in the dimeric form, which is named RCC2 (Dekker and Boekema, 2005, Figure 1.3). The PSII dimeric structure has been clarified from two thermophilic cyanobacteria, *Thermosynechococcus elongatus* and *Thermosynechococcus vulcanus*, at resolutions of 3.8-2.9 Å. Both the structure data and biochemical results approve two PSII monomers to be connected by several low molecular weight subunits located on their surface (Kawakami et al., 2011b). Various protein factors are involved in the formation and stabilization of PSII dimers, which will be introduced in the following section.

With recent progresses by single particle electron microscopy, atomic force microscopy, and tomographic reconstruction of intact and fragmented chloroplasts, the supramolecular organization of PSII was studied. A variable amount of peripheral antenna proteins associate with dimeric PSII core complexes to form PSII-LHCII supercomplexes. For example, a study on spinach demonstrated that C_2 (dimeric PSII core center) associate firstly with two LHCII S-trimers (strongly binding trimer) together with two copies of CP29 (Lhcb4), CP26 (Lhcb5) extending to a C_2S_2 supercomplex, and then two M-trimers (medium strength binding) bind to C_2S_2 together with two copies of CP24 (Lhcb6) to achieve a $C_2S_2M_2$ supercomplex. Furthermore, there are also LHCII L-trimers loosely bound to the supercomplex, which is only present in certain species (reviewed in Dekker and Boekema, 2005).

1.2.1.3 Proteins involved in the assembly and sustenance of PSII

Nowadays, besides structural studies, lots of efforts have also been put on the identification and analysis of protein factors involved in PSII assembly and stabilization. These factors could either constructively or transiently participate in PSII formation. Transiently involved auxiliary proteins, which are mostly encoded by the nucleus, are not found in functional PSII complexes. Among all the assembly factors which have been characterized till now, several are highly conserved in cyanobacteria and chloroplasts. For example, Hcf136 (also termed Ycf48), of which homologs are found in both *Arabidopsis thaliana* and *Synechocystis* sp. PCC 6803, functions in PSII assembly and stabilization (Komenda et al., 2008; Meurer et al., 1998). Its binding site on the PSII reaction center and its 3D structure has also been determined recently (Komenda et al., 2008). There are still many conserved nucleus-encoded proteins with potential function on PSII complex formation, whose exact role needs to be clarified in the future. One protein family thought to be involved in PSII assembly is represented by light-harvesting-like (LIL) proteins which will be introduced in section 1.2.1.3.1.

In addition, several PSII low molecular weight (LMW) subunits, encoded by the nucleus or chloroplast, were found to be involved in PSII assembly or stabilization. For example, the LMW proteins Psb27, Psb28 and Psb29 were identified as substoichiometric components associating with PSII RC47 sub-complexes to form the final active PSII complexes (Kashino et al., 2002). More LMW proteins required for assembly or stabilization of PSII complexes, especially PSII dimers, will be introduced in section 1.2.1.3.2.

1.2.1.3.1 LIL (light-harvesting-like) proteins - auxiliary factors involved in PSII assembly

Although, compared to structural PSII subunits, the detection of auxiliary proteins which are normally low abundant or only transiently expressed, is difficult, several assembly factors have been identified, which are proposed to play a role in pigment binding and assembly. Among those factors are members of several famous protein families, like the ALB (albino) proteins, which were thought to be involved in LHCII assembly in both *A. thaliana* and *C. reinhardtii*, as well as the LPA (low PSII accumulation) family, which was reported to function during the assembly of the chlorophyll binding protein CP43 (Cai et al., 2010; Göhrea et al., 2006). Since the assembly of pigments seems to play a role for the entire PSII assembly process, the members of the LIL (light-harvesting-like protein) family attracted more attention nowadays.

LIL proteins are stress induced short-lived proteins with low molecular mass, located in thylakoid membranes of chloroplasts as well as plasma membranes of cyanobacteria. Protein sequence analyses indicate that LIL proteins share similar sequences with LHCII of higher plants with conserved chlorophyll binding residues. The LIL proteins consist of three groups: (I) three-helix ELIPs (one super protein family called early light-induced proteins); (II) two-helix SEPs (stress-enhanced proteins) and (III) one-helix HLIPs (high-light-induced proteins), including OHP (one-helix proteins) and SCPs (small chlorophyll *a*/*b*-binding-like proteins) in prokaryotic organisms (Adamska et al., 2001). Although being able to bind pigments, LIL proteins do not have functions in light energy harvesting and their precise roles are only beginning to be elucidated (Mulo et al., 2008). In *A. thaliana*, the amount of *ELIP* transcripts and proteins increases depending on the light intensity (Heddad et al., 2006). It was also described that the accumulation of AtELIP1 and carotenoid biosynthesis related (CBP) proteins in green algae starts right after the increase of photodamaged PSII centers (Hutin et al., 2003; Jin et al., 2003; Jin et al., 2001). Hence, the LIL proteins were speculated to play a protective function in the thylakoid membranes by binding free chlorophylls which are released during photoinhibition. Alternatively, they could be involved in the assembly of pigment-protein complexes (Hutin et al., 2003).

As described above, LIL proteins are conserved in many photosynthetic organisms, for instance, light induced one-helix proteins have been found in cyanobacteria, green algae and higher plants, but their exact functions, especially on PSII assembly, still require further characterization.

1.2.1.3.2 Low molecular weight proteins

From the high resolution data of the 3D PSII structure, the presence of many low molecular weight (LMW) proteins is observed, which are encoded either by the nucleus or chloroplast genome. More than half of the LMW proteins are less than 15 kDa, and most of them consist of a single transmembrane α-helix (reviewed in Shi et al., 2012). As mentioned above, some of these LMWs function as assembly or stabilization factors for PSII complexes, which were firstly speculated by their structure model, and then confirmed by biochemical analyses.

Besides LMW proteins referred above, like Psb27, Psb28 and Psb30, there are more small PSII subunits approved to be PSII assembly factors. For instance, in most oxygenic phototropic organisms, the *psbEFLJ* operon encodes four small subunits PsbE, PsbF, PsbL and PsbJ, among which, PsbE and PsbF are involved in the early steps of PSII assembly. Consequently *PsbE* and *PsbF* deletion mutants from *C. reinhardtii* and tobacco are not able to

perform photoautotrophic growth (Morais et al., 1998; Pakrasi et al., 1991; Suorsa et al., 2004; Swiatek et al., 2003). Furthermore, *PsbL* deletion mutants from *Thermosynechococcus elongatus* and tobacco do not assemble detectable PSII dimers, whereas PsbJ is involved in the assembly of the water splitting complex (Ohad et al., 2004; Suorsa et al., 2004; Swiatek et al., 2003). Apparently, there are various functions performed by LMW proteins in PSII complex assembly or stabilization, as well as photoprotection, electron transfer and so on, which could be deduced from mutant phenotypes. A summary of respective mutant phenotypes in cyanobacteria and eukaryotes and proposed functions of corresponding LMW proteins are given in Table 1.1, which is mainly focusing on PSII dimer formation or stabilization.

There are still increasing amounts of LMW proteins being found in both prokaryotes and eukaryotes. But similar to LIL proteins, their precise subcellular localizations and biological functions leave researchers a large space to explore.

Table 1.1 LMWs involved in PSII dimer formation or stabilization and corresponding mutant phenotypes.

Protein	Prokaryotic mutants (cyanobacteria)	Eukaryotic mutants (Arabidopsis/ Chlamydomonas/ Tobacco)	Function	References
PsbI	photoautotrophic growth; less oxygen evolution; light sensitivity; no PSII dimers	photoautotrophic growth under low light; less oxygen evolution; dramatically reduced PSII dimers; light sensitivity	PSII dimerization/stabilization; maintenance of PSII structure and function under high light	Ikeuchi et al., 1991; Künstner et al., 1995; Schwenkert et al., 2006
PsbK	photoautotrophic growth; low electron transport	no photoautotrophic growth; only 10% of PSII left; no PSII activity	plastoquinone binding; PSII stabilization	Ikeuchi et al., 1991; Iwai et al., 2010; Takahashi et al., 1994
PsbL	no photoautotrophic growth; no oxygen evolution	no photoautotrophic growth; no or reduced photosynthetic activity; no PSII dimers	donor side electron transfer; PSII stabilization	Anbudurai and Pakrasi, 1993; Luo and Eaton-Rye, 2008; Swiatek et al., 2003
PsbM	light sensitivity; rapid photoinactivation; less PSII dimers	light sensitivity; reduced phosphorylation of D1 and D2	PSII dimerization	Kawakami et al., 2011a; Umate et al., 2007
PsbH	slower photoautotrophic growth; low oxygen evolution; no PSII dimers	no PSII dimers, no PSII activity	PS II dimerization	Iwai et al., 2006; O'Connor et al., 1998
PsbTc	photoautotrophic growth; normal oxygen evolution; less PSII dimers	photoautotrophic growth; light sensitivity	recovery of photodamaged PSII; PSII dimerization /stabilization	Bentley et al., 2008; Iwai et al., 2004; Ohnishi et al., 2007; Ohnishi and Takahashi, 2001, 2008
PsbW	no homologue	photoautotrophic growth; no PSII dimers; light sensitivity ; slower recovery from photoinhibition	PSII dimerization; photoprotection	Boekema et al., 2000; García-Cerdán et al., 2011; Shi et al., 2000; Thidholm et al., 2002
Psb30	photoautotrophic growth; reduced oxygen evolution under high light; less PSII dimers	no mutant available	indirect PSII dimer stabilization	Inoue-Kashino et al., 2008; Sugiura et al., 2010
Psb32	severe photoinhibition; slower recovery rates	light sensitivity; more PSII monomers and less PSII dimers	functions in PSII repair cycle; PSII dimerization	Mulo et al., 2008; Sirpiö et al., 2007; Wegener et al., 2011

1.3 Chloroplast gene expression

In higher plants, two distinct RNA polymerases (RNAP), the plastid-encoded plastid RNA polymerase (PEP) and the nuclear-encoded plastid RNA polymerase (NEP) are involved in transcription of chloroplast genes (reviewed in Borner et al., 2011). The PEP polymerase is verified to have a cyanobacterial origin (Navarro et al., 2000; Pfannschmidt and Link, 1997; Severinov et al., 1996). The recognition of promoters by the PEP polymerase is mediated by nucleus-encoded differentially expressed sigma-like transcription factors (SLF) (Isono et al., 1997; Little and Hallick, 1988; Suzuki et al., 2004). The activated PEP complex contains several accessory proteins encoded by nuclear genes, which shows that the nuclear genome has an obvious impact on the regulation of chloroplast genome transcription (Pfalz et al., 2006; Pfannschmidt et al., 2000; Suzuki et al., 2004). The other RNA polymerase, NEP, is a single polypeptide chain encoded in the nucleus, similar to the mitochondrial RNAP of yeast, and RNAPs from bacteriophages T7, T3, and SP6 (reviewed in Cahoon and Stern, 2001; Liere et al., 2011). It is thought that both RNA polymerases act cooperatively in plastid transcription. NEP is primarily responsible for transcribing genes encoding proteins of the plastid genetic machinery and PEP genes, whereas PEP is mainly responsible for transcribing photosynthesis-related genes.

Interestingly, the NEP polymerase is not present in algae, like *C. reinhardtii*, *Ostreococcus* and *Thalassiosira* (Armbrust et al., 2004; Derelle et al., 2006). All attempts to obtain algae mutants with disruptions of PEP subunit encoding genes failed, which demonstrated that all chloroplast genes of *C. reinhardtii* are likely transcribed by PEP (Fischer et al., 1996; Smith, 2002).

The products of both polymerases are typically polycistronic transcripts. Most primary transcripts need extensive splicing, endonucleolytic cleavage, 5’ and 3’- end maturation, and/or editing (Monde et al., 2000). The plastid genomes in plants and algae contain numerous introns, defined as group I or group II. The *C. reinhardtii* chloroplast has 5 group I and 2 group II introns, whereas plants have ~ 17 groups II and only 1 group I intron (reviewed in Herrin and Nickelsen, 2004). The processing of both intron classes is under various regulations, for instance, the *psbA* mRNA splicing in *C. reinhardtii* is dependent on light variations, and moreover, most chloroplast transcripts are predominantly unspliced in leaf meristems and roots. (Barkan, 1989; Deshpande et al., 1997).

The translation of chloroplast genes is performed by a prokaryote-like translation apparatus, consisting of a 70*S* ribosome that contains 23*S*, 16*S* and 5*S* rRNAs, which is different from the cytosolic 80*S* ribosome (Manuell et al., 2004; Trempe and Glitz, 1981). The general

mechanism of translation is closely related to the involvement of regulatory factors. These regulators play important roles during translation initiation, elongation, and stabilization and will be introduced in the following chapter.

1.3.1 Regulation of chloroplast gene expression

Chloroplasts retained their own gene expression machinery, but given that chloroplast proteins are encoded in two separate genomes, a coordinated expression is required to produce correct amounts of organellar proteins and support their functions. Due to the limited number of chloroplast encoded proteins, this coordination mostly relies on nucleus-encoded regulators.

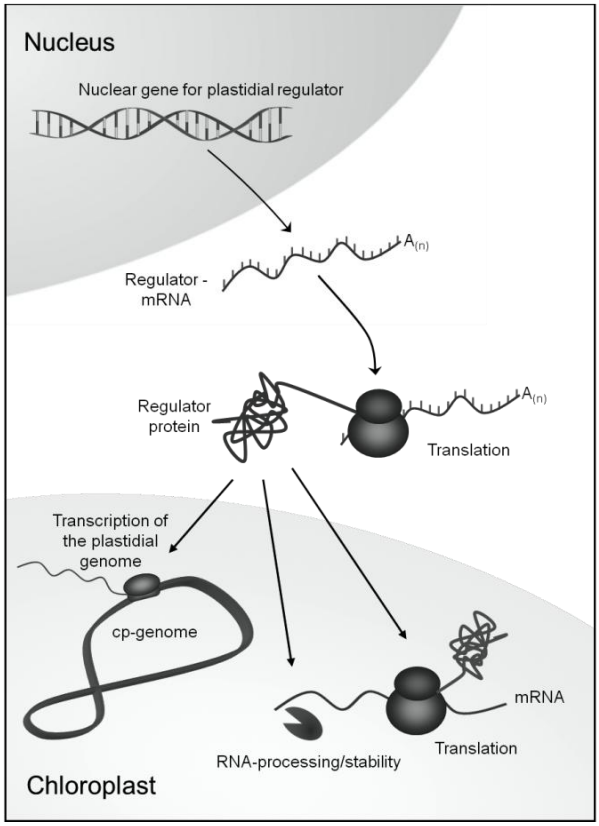


Figure 1.4 Regulation of chloroplast gene expression by nucleus-encoded regulators (adapted from Bohne et al., 2009). For further explanation, see text.

Increasing numbers of nuclear mutants with disrupted chloroplast gene expression have led to the identification of many genes whose products either directly or indirectly participate in protein expression processes, i.e. transcription, post-transcriptional processes, and translation (Figure 1.4). Regulatory proteins acting at different levels of chloroplast gene expression as well as internal and external signals influencing these processes are described in more detail in the following sections.

1.3.1.1 Transcriptional regulation

Beside the above mentioned sigma-like factors, only a few nuclear gene products have been revealed to be involved in plastid transcription. In mature plant chloroplasts, the transcription rates of genes encoding the reaction center proteins of PSI and PSII are controlled by the redox state of the plastoquinone pool (Pfannschmidt et al., 1999). Two kinases, STN7 (on thylakoid membranes) and CSK (in the stroma) in *A. thaliana* chloroplasts are thought to influence chloroplast transcription in a redox-dependent manner (Bonardi et al., 2005; Pesaresi et al., 2009; Puthiyaveetil et al., 2008). Moreover, the accumulation of chloroplast transcripts seems to be dependent on light quality and quantity as well as the developmental stage of the plastid (Emanuel et al., 2004; Link et al., 1996; Mayfield et al., 1995; Mullet, 1993; Rapp et al., 1992; Zoschke et al., 2007). For instance, in barley, *psbD-psbC* transcript accumulation is induced by blue light, but neither by red nor by far-red light (Gamble and Mullet, 1989).
However, transcriptional regulation seems to play only a secondary role and most regulation of chloroplast gene expression is observed at subsequent levels.

1.3.1.2 Posttranscriptional regulation

Most regulations of chloroplast gene expression are post-transcriptional. These processes are controlled by nucleus-encoded proteins, which are also named as post-transcriptional regulators of organelle gene expression (ROGEs). ROGEs function in two typical classes of regulation: one is required for the maturation (mRNA processing, splicing and editing) and/or stabilization of organellar transcripts, the other one is involved in translation (translation initiation, elongation and stabilization) of organellar transcripts (Raynaud et al., 2007).

1.3.1.2.1 Transcript maturation and stabilization

It was demonstrated that translation of individual mRNAs usually needs processed, shorter transcripts. The processing of chloroplast mRNA is a two-step mechanism: endonucleolytic cleavage, and exonucleolytic processing (Monde et al., 2000). This process in land plants and *C. reinhardtii* typically works at the 5’ ends of mRNA, which was first reported for the *psbA* mRNA processing in *C. reinhardtii* and then *psbB* and *psbD* (Bruick and Mayfield, 1998; Nickelsen et al., 1999; Vaistij et al., 2000). The same phenomenon was also observed in land plants. For instance, the maize *chloroplast RNA processing 1* (*crp1)* gene, encoding a pentatricopeptide repeat protein (PPR), is required for cleaving *petD* coding sequences from a

polycistronic precursor. Furthermore, the *A. thaliana* HCF107 protein is necessary for obtaining *psbH* transcripts with fully processed 5' termini (Barkan and Goldschmidt-Clermont, 2000; Barkan et al., 1994). It was reported that the unsuccessful cleavage of mRNAs leads to loss of corresponding proteins, which suggest that 5' processing of chloroplast mRNAs assists to increase the translational efficiency. The detailed processing mechanism is still under investigation, including 3' termini processing and protection of RNA from the action of RNases. It is widely accepted that processing events are regulated by the coordination of several factors, among which nucleus-encoded regulators play essential roles. A summary of nuclear encoded stabilization and maturation factors identified in *C. reinhardtii* is given in Table 1.2.

The next step for mRNA maturation is splicing of transcripts, either *cis*-splicing or *trans*-splicing, which is also under the control of nuclear regulators (reviewed in Herrin and Nickelsen, 2004). For instance, at least 14 nuclear gene products required for *psaA* trans-splicing have been found in *C. reinhardtii* (Goldschmidt-Clermont et al., 1990). Several of them were reported to be involved in the splicing of both types of introns. However, most regulators function specifically in the splicing of either intron I or II. Interestingly, most of the regulators have their specific targets, while only a few examples seem to fulfill a more general functions (Balczun et al., 2006; Glanz et al., 2006; Kroeger et al., 2009; Merendino et al., 2006; Ostersetzer et al., 2005; Williams-Carrier et al., 2008). In *C. reinhardtii*, research about group I intron splicing is very limited, on the contrary, genetic analyses of group II intron splicing in chloroplasts have been more fruitful (Perron et al., 2004).

In land plants, the editing of chloroplast RNA nucleotides from cytidine to uracil residues is another important maturation step, which also requires regulators encoded by nucleus, whereas no editing of RNAs is known to occur in green algae like *C. reinhardtii*. Most editing sites are located in reading frames except a few in non-coding regions (reviewed in Stern et al., 2010). *Trans*-acting factors bind to transcripts via *cis*-element adjacent to the editing site to facilitate access of an unidentified RNA-editing enzyme. Several members of the pentatricopeptide repeat protein family have been characterized to be essential for RNA editing in *A. thaliana* (reviewed in Small and Peeters, 2000).

The translation rate of mature chloroplast mRNAs also highly relies on RNA stability, which could be influenced by decay pathway. Three processes contribute to the degradation of transcripts by various RNases: endonucleolytic cleavage, polyadenylation, and exonucleolytic decay, among which, the first one is the rate-limiting step (reviewed in Stern et al., 2010). Various regulator proteins are required to protect mRNAs from endonucleolytic and exonucleolytic degradation, and most of them are repeat proteins, such as TPR and PPR,

which often protect 5' termini of mRNAs from exonuclease degradation (Boudreau et al., 2000; Loiselay et al., 2008). More details will be introduced in the later chapters.

1.3.1.2.2 Translational regulation

Numerous nucleus-encoded regulators have been found to function during chloroplast protein synthesis (Figure 1.5), which include certain plastid ribosomal proteins, initiation factors, elongation factors and tRNA synthetases (reviewed in Harris et al., 1994).

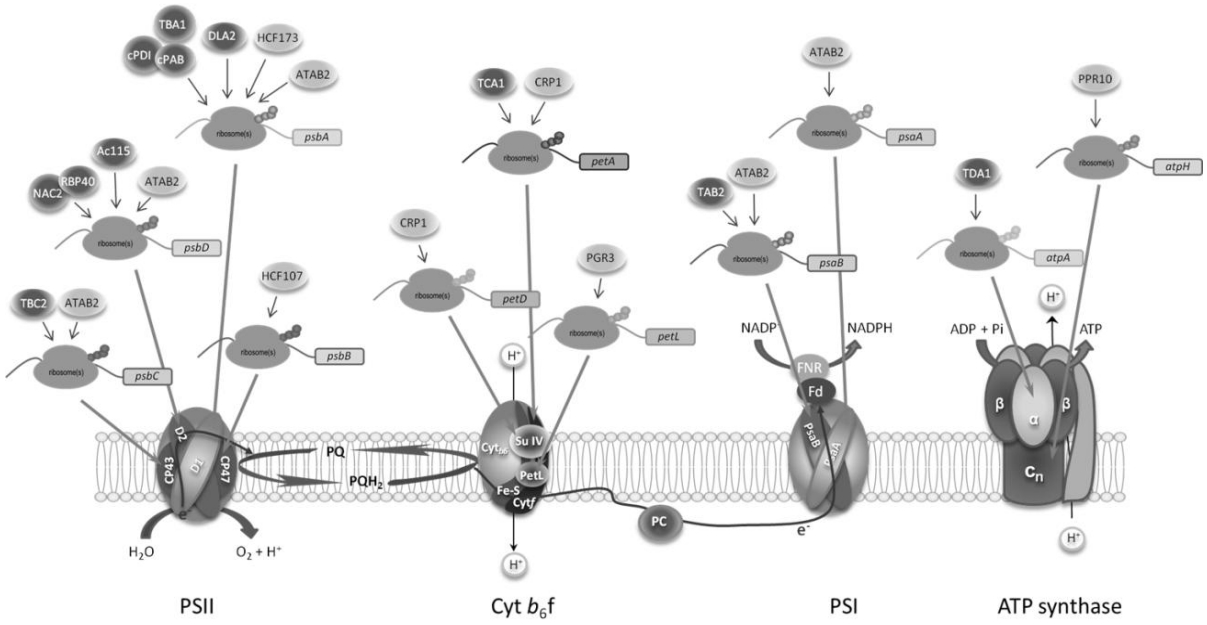


Figure 1.5 Cloned nucleus-encoded factors involved in the translation of thylakoid proteins.
Regulators in dark grey were characterized in *C. reinhardtii*, the ones in light gray in higher plants (Figure kindly provided by A. Bohne).

It is worth noticing that, unlike prokaryotic RNA translation, the Shine-Dalgarno (SD) sequence is not always necessary for eukaryotic ribosome binding. It was observed that a lack of SD sequences can be compensated by sequence specific factors guiding the ribosomes to their sites of action (Hirose and Sugiura, 1996). *In vitro* and *in vivo* approaches were applied on wild-type and 5' UTR mutants to reveal translational elements in both tobacco and *C. reinhardtii* (Manuell et al., 2004; Yukawa et al., 2007). Till now, the 5' UTR region of chloroplast mRNA is verified to be the translation initiation site, by means of biochemical approaches.

Several nuclear gene products were identified, which influence chloroplast translation. For instance, CRP1 from maize, and HCF107 and HCF173 from *A. thaliana* are required for *petA/petD*, *psbB* and *psbA* mRNA translation, respectively (Sane et al., 2005; Schmitz-Linneweber et al., 2005; Schult et al., 2007). In *C. reinhardtii*, the most predominant even though controversially discussed example is the regulation of D1 protein synthesis, which is dependent on a multi-subunit complex (Harris, 2009; Manuell et al., 2004; Uniacke and Zerges, 2009). By affinity chromatography using the *psbA* 5' UTR as ligand, four subunits were isolated of this complex, which includes a 63 kDa protein disulfide isomerase (cPDI), a 47/70 kDa poly (A)-binding protein (cPAB1), a 55 kDa protein (RB55), and RB38. Furthermore, TBA1, an oxidoreductase was described to control the D1 synthesis via redox regulation in *C. reinhardtii*. In addition, an independent RNA binding protein of 63 kDa (RBP63) was identified to bind to an adenosine-rich region upstream of the *psbA* start codon (Ossenbühl. et al., 2002). On the other hand, D1 synthesis seems not to be controlled by its 5' UTR during recovery from photoinhibition (Minai et al., 2006). Another example for translational regulation is the D2 synthesis, where the tetratricopeptide repeat (TPR) protein Nac2 and the RNA binding protein RBP40 coordinately function in the alteration of *psbD* mRNA secondary structure and translation initiation.

It is necessary to notice that in *C. reinhardtii*, a further level of translational regulation named CES (control by epistasy of synthesis) principle was described these years, which controls the translation and assembly of photosynthesis complexes. Under this CES principle, complex assembly starts firstly from insertion of an anchor protein (also called dominant subunit), which acts as a scaffold for following assembly steps. For instance, D2 is the anchor protein for PSII, PetB for Cyt b_6f, and PsaB for PSI. In the absence of these proteins, translation of the next protein to be inserted in the complex is inhibited (Choquet and Vallon, 2000). In terms of that, within the same complex, translation of specific chloroplast mRNAs might be influenced by translation deficiency of another mRNA via feedback mechanisms.

From all the information above, it is readily identifiable that *C. reinhardtii* as one the most predominant model organisms, was highly employed for the characterization of nucleus-encoded factors which are involved in chloroplast gene expression. Therefore, a comprehensive summary of cloned factors involved in regulation of chloroplast gene expression in *C. reinhardtii* is given below in Table 1.2.

Table 1.2 Cloned nucleus-encoded regulatory factors involved in chloroplast gene expression in *C. reinhardtii*

Factor	Homology	Target	Reference
Transcription factors:			
RpoD (Sig1)	Sigma factor	RNA-polymerase	Bohne et al., 2006; Carter et al., 2004
RNA stability factors:			
Mbb1	TPR-protein	*psbB*	Vaistij et al., 2000
Nac2	TPR-protein	*psbD*	Boudreau et al., 2000
Mca1	PPR-protein	*petA*	Loiselay et al., 2008
Mcd1	OPR-protein	*petD*	Murakami et al., 2005
MRL1	PPR-protein	*rbcL*	Johnson et al., 2010
RNA processing factors:			
Raa1	OPR-protein	*psaA*	Merendino et al., 2006; Perron et al., 2004
Raa2 (Maa1)	Pseudouridin-Synthetase	*psaA*	Perron et al., 1999
Raa3	Pyridoxamine-5-phosphate oxidase	*psaA*	Rivier et al., 2001
Raa4	-	*psaA*	Glanz et al., 2012
Rat1	Poly (ADP-ribose)-polymerase	*tscA 3'*	Balczun et al., 2005
Rat2	-	*tscA 3'*	Balczun et al., 2005
Translation factors:			
Tba1	Oxidoreductase	*psbA*	Somanchi et al., 2005
cPAB1	Poly (A)-binding protein	*psbA*	Yohn et al., 1998
cPDI	Protein disulfide isomerase	*psbA*	Kim and Mayfield, 1997
RBP40 (RB38)	-	*psbD*	Schwarz et al., 2007
Tca1	-	*petA*	Raynaud et al., 2007
DLA2	E2 subunit pyruvate dehydrogenase	*psbA*	Bohne and Nickelsen, unpublished
TBC2	OPR protein	*psbC*	Auchincloss et al., 2002
NAC2	TPR protein	*psbD*	Boudreau et al., 2000
AC115	-	*psbD*	Rattanachaikunsopon et al., 1999
TAB2	ATAB2	*psaB*	Dauvillee et al., 2003
TDA1	OPR protein	*atpA*	Eberhard et al., 2011

1.3.2 Nucleus-encoded repeat protein families involved in the regulation of chloroplast gene expression

In the last chapter, several nucleus-encoded regulators have already been introduced. Mostly, these regulatory factors belong to either pioneer proteins which are not conserved among eukaryotes, or protein families which are defined by their tandem motif repeats (compare Table 1.2). Moreover, these repeat protein families were verified to be involved in protein-

protein or protein-RNA interaction. So far, tetratricopeptide repeat (TPR), pentatricopeptide repeat (PPR), and octotricopeptide repeat (OPR) proteins have been characterized and are described below.

1.3.2.1 TPR proteins

TPR proteins are studied extensively among three repeat protein families, with anti-parallel α-helices TPR motifs which generate a helical structure with an amphipathic character (Blatch and Lassle, 1999; Sikorski et al., 1990). The TPR repeat structure has been confirmed by crystallization for several cases with an example of the protein phosphatase 5 shown in Figure 1.6 (reviewed in D'Andrea and Regan, 2003; Das et al., 1998).

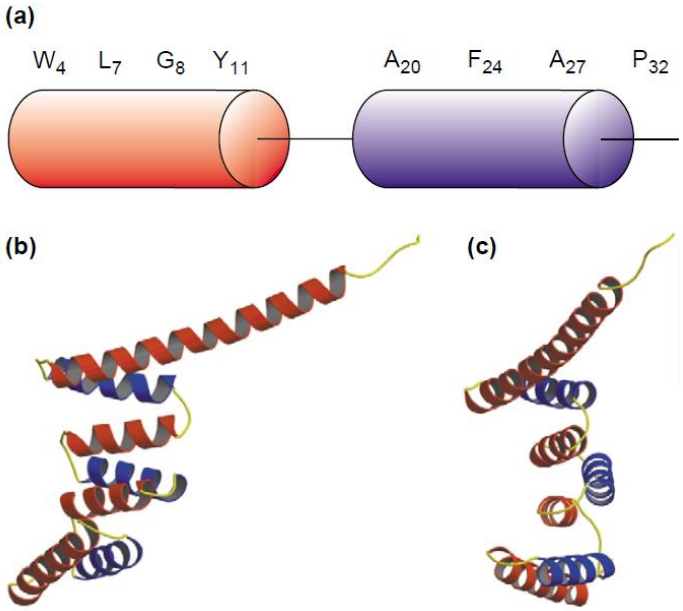


Figure 1.6 Structure of a tetratricopeptide repeat (TPR) motif (adapted from D'Andrea and Regan, 2003). **a**: Schematic representation of the secondary structure of 34 amino acids in TPR motif with original consensus sequence shown above the helices. Helix A, helix B, and the loop region are shown in red, blue and black, respectively. **b:** Front and **c:** perpendicular views of the three TPRs of protein phosphatase 5.

The TPR motif consists of 34 degenerate amino acid residues, arranged in 3-16 tandem repeats. Only a few positions of these residues are conserved. The structure of the TPR domain was studied intensively in the past. Using mutagenesis on specific amino acids within the TPR domain revealed highly conserved residues to be mainly responsible for structure maintenance, whereas the other non-conserved ones are related to specific protein functions (Letunic et al., 2002; Prapapanich et al., 1996). TPR proteins are highly conserved in all the organisms, such as bacteria, fungi, plants, insects, animals, and humans. There are no

significant differences between prokaryotic and eukaryotic TPR domains (Blatch and Lassle, 1999). Moreover, they are verified to participate in various processes via protein-protein interactions, such as RNA processing, cell cycle, protein folding as well as protein transporting (reviewed in D'Andrea and Regan, 2003).

The first TPR protein named $nuc2^{+}$ was characterized in yeast, involved in the cell division cycle (Hirano et al., 1990; Sikorski et al., 1990). Till now, 22-26 TPR proteins have been identified by sequence analysis in Synechocystis, one example of which is PratA, involved in the maturation process of the D1 protein (Klinkert et al., 2004; Schottkowski et al., 2009a). Pitt is another TPR in Synechocystis, necessary for the early steps of photosynthetic pigment/protein complex formation (Schottkowski et al., 2009b). In *C. reinhardtii*, the nucleus-encoded Nac2 protein functions in stabilizing the *psbD* transcript, together with other protein partners (Boudreau et al., 2000; Schwarz et al., 2007). The above mentioned orthologous proteins Hcf107 in *A. thaliana* and MbbI in *C. reinhardtii* are two other TPR proteins responsible for *psbH* and *psbB* transcript stability, respectively (Felder et al., 2001; Vaistij et al., 2000). The *A. thaliana* LPA1 is another prominent TPR protein involved in PSII assembly; however its homolog in *C. reinhardtii*, REP27, is considered to participate in the repair cycle of PSII (Park et al., 2007; Peng et al., 2006).

The well-defined TPR profile and complete sequencing of several model organism genomes provide possibilities to predict members of the entire TPR-protein family. However, their interaction partners as well as precise molecular working modes require further verification.

1.3.2.2 PPR proteins

The PPR (pentatricopeptide repeat) proteins belong to another group of repeat protein, with presence of degenerate 35 amino acid motif, repeated in up to 30 tandem (Small and Peeters, 2000). The PPR proteins are also predicted to contain an array of α-helices, which classify them as a member of the "α-solenoid" super family together with TPRs (Small and Peeters, 2000). PPR proteins were first characterized in *A. thaliana*, followed by continuing identifications in different organisms. The PPR proteins are widely distributed in eukaryotes, especially in plants, but not in prokaryotes. For instance, there are approximately 450 PPRs in *A. thaliana* and the number increases to 600 in both poplar and Vitis. However, the number of PPRs is significantly reduced in algae, only 11 in *C. reinhardtii* (Schmitz-Linneweber and Small, 2008).

Genetic studies together with biochemical identifications of PPR proteins revealed that they are required for a wide range of post-transcriptional regulations, via their involvement in

organellar RNA metabolism. A domain-swap experiment between *A. thaliana* PPR proteins CRP1 and CRR4 demonstrated that PPR domains are responsible for RNA binding (Okuda et al., 2007). Therefore, PPRs were inferred to participate in various steps of RNA metabolism. For example, an RNA splicing function was approved for the chloroplast OTP51 protein from *A. thaliana*, which is required for splicing of the second intron of *ycf3* mRNA (de Longevialle et al., 2008). Moreover, the PPR4 protein in maize is responsible for *trans*-splicing of intron of the *rps12* mRNA (Schmitz-Linneweber et al., 2006). In addition, PPR's RNA editing functions were also observed in plants, for instance, the above mentioned *A. thaliana* CRR4 and CRR21 proteins are required for the edition of the *ndhD* transcript (Kotera et al., 2005; Okuda et al., 2007). Moreover, some PPR proteins are essential for organellar transcripts stabilization. Taken *C. reinhardtii* as an example, two characterized PPRs, MCA1 and MRL1, were proved to participate in the stabilization of *petA* and *rbcL* mRNAs, respectively (Johnson et al., 2010; Loiselay et al., 2008). The same function is also reported in higher plants. The *A. thaliana* PGR3 protein stabilizes *petL* transcripts, and PPR5 in maize stabilizes *trnG-UCC* precursor (Beick et al., 2008; Yamazaki et al., 2004). On the other hand, some PPRs perform a completely opposite function to stabilization by being a processing factor, such as CRP1 protein in maize, which is an endonuclease itself and required for the processing of the *petD* mRNA from a polycistronic precursor (Fisk et al., 1999; Schmitz-Linneweber et al., 2005). A translation activation function is also found for several PPRs, such as the above mentioned maize CRP1, which initiates the translation of *petA* and *psaC* mRNA, and *A. thaliana* CRR2 protein, essential for the expression of chloroplast *ndhB* (Fisk et al., 1999; Hashimoto et al., 2003; Meierhoff et al., 2003; Schmitz-Linneweber et al., 2005). One special example also shows PPR's involvement in the coordination of chloroplast and nucleus gene expression. At this, GUN1 (genomes uncoupled 1), is involved in retrograde signaling from the chloroplast to the nucleus (Koussevitzky et al., 2007).

1.3.2.3 OPR proteins

OPR (octotricopeptide repeat) proteins were characterized recently as a new α-solenoid super family, like TPRs and PPRs, with degenerate 38-40 amino acid repeats (Eberhard et al., 2011). Although the structure of the OPR motif is still unclear, the secondary structure prediction speculated super helical motifs formed by arrayed α-helices (Eberhard et al., 2011).

Bioinformatic analyses show that OPR proteins are distributed quite differently among all the organisms. There are more than 100 OPR proteins present in *C. reinhardtii* with predicted organellar localizations, whereas only one single OPR was found in *A. thaliana* (O. Vallon, A.

Bohne, L. Cerutti, J. D. Rochaix, unpublished data). Based on the high number of OPRs found in *C. reinhardtii*, a logo plot of OPR motifs occurring in this alga is given in Figure 1.7. Frequently emerging residues within the motif are represented by LWALA at positions 11-15 and a previously described PPPEW motif (positions 22-27; Eberhard et al., 2011).

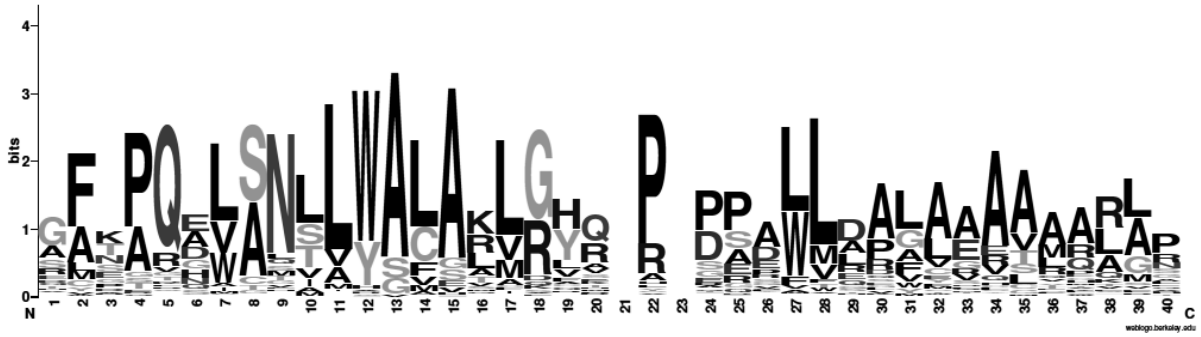


Figure 1.7 Logo plot of the over-represented motif from OPR repeats found in *C. reinhardtii.*
The height of each nucleotide is shown proportional to its frequency, with the most common nucleotides on top (O. Vallon, A. Bohne, L. Cerutti, J. D. Rochaix, unpublished data).

The newly identified OPR proteins are thought to play roles during RNA metabolism. Till now, three OPR proteins from *C. reinhardtii* were characterized to participate in RNA processing. For example, RAT2, localized in the chloroplast stroma, is clarified to be involved in the 3' end processing/maturation of *tscA* (Balczun et al., 2005). The *tscA* RNA is a co-factor, which participates in *trans*-splicing of intron I of *psaA* mRNA. In addition, RAA1 is another OPR protein found in a large ribonucleo-protein complex, which is involved in *trans*-splicing of both intron I and intron II of the *psaA* mRNA. Unlike RAT2, RAA1 was found in thylakoid membranes. Two functional domains were found in RAA1, one in the C-terminus, responsible for splicing of intron I, and the other in the central part needed for *trans*-splicing of intron II (Merendino et al., 2006). Moreover, recently MCD1 has also been considered to be an OPR protein. The MCD1 protein was approved to interact with the 5' UTR of *petD* mRNA and protect this transcript from degradation by 5' exoribonucleolytic cleavage (Murakami et al., 2005). Besides the regulation of RNA processing, OPR proteins also play essential roles during translation processes. The TBC2 protein, possessing nine OPR motifs, was shown to be part of a large protein complex (~ 400 kDa), localized in chloroplast stroma fraction, where it is involved in CP43 translation via interaction with the 5'UTR of the *psbC* mRNA (Auchincloss et al., 2002). Another translational regulator is TDA1, with OPR repeats present

at the C-terminus, which is described to participate in the translation of *atpA* transcripts (Eberhard et al., 2011).

All evidences mentioned above show that the members of the OPR repeat protein family play diverse and important roles during post-transcriptional regulation via their participation in RNA metabolisms.

1.4 Model organisms: *Arabidopsis thaliana* and *Chlamydomonas reinhardtii*

In land plants, most genetic studies of nuclear genes involved in plastid gene expression have been carried out in *Arabidopsis thaliana*, which offers well developed genetic tools and abilities to clone nuclear genes defined by mutations. *A. thaliana* possesses only a small nuclear genome of 157 Mb, which facilitates genetic mapping. Meanwhile, a huge *A. thaliana* T-DNA insertion mutant collection gives convenient support for research. Although *A. thaliana* offers advantages of nuclear transformation, the plastid transformation is still not available. In addition, sugar dependent growth of *A. thaliana* mutants causes complications for phenotype analysis and biochemical approaches. Therefore, alternative model organisms are required (Sheen, 1999).

Chlamydomonas reinhardtii, as one of the outstanding model organisms, is an unicellular green alga, with the ability to grow heterotrophically in the acetate-containing media, where it still assembles fully functional thylakoid membrane complexes (reviewed in Nickelsen and Kück, 2000). The structure specialties include a single cup-shaped chloroplast containing the photoreceptive “eye spot”, which allows the cell to perform phototaxis, and the pyrenoid, where the carbon dioxide fixation and protein synthesis happen, including PSII assembly (Harris, 2001; Uniacke and Zerges, 2008). In addition, structure and function analyses of flagella, light perception, cell-cell recognition and cell cycle control also attract lots of attention (Harris, 2001). Advantageous features of *C. reinhardtii* include the ease of obtaining and maintaining non-photosynthetic mutants, and the well-developed technologies for transforming both nuclear and plastid genomes (Rochaix, 1995). Meanwhile, both the availability of the complete genome sequence of *C. reinhardtii* and BAC (bacteria artificial chromosome) clones, which cover the respective genomic regions for complementation, let *C. reinhardtii* face a bright future as a model system (Meslet-Cladiere and Vallon, 2011).

1.5 Aims of this study

Photosynthesis is the most studied field in plant sciences. However, the exact working modes of already described proteins involved in chloroplast gene expression are often still little understood. Additionally, many yet unknown nucleus encoded proteins are speculated to perform important roles in the expression and assembly of photosynthesis-related complexes.

As photosystem II is considered to represent the key protein complex of photosynthesis light reactions, the present study was designed to extend the knowledge of factors participating in the expression and assembly of PSII subunits. Therefore, 16 nuclear insertion mutants from the green alga *C. reinhardtii* revealing a PSII deficient phenotype were used in a forward genetic approach to identify potentially new candidate proteins. Following an initial phenotypical characterization, promising mutants were chosen for the identification of mutated genes causing the PSII phenotype by a PCR based approach. For five out of eight mutants the corresponding mutated gene could be identified. Two of them, possessing insertions in the *OHP2* or *CLR24* gene, respectively, were selected for a detailed molecular and phenotypical characterization to elucidate the role of encoded proteins in photosynthesis. Whereas OHP2 (one helix protein 2) represents a member of the light-harvesting-like (LIL) protein family, with one predicted transmembrane region, CLR24 belongs to the newly found α-solenoid OPR super family, which is thought to have diverse functions during post-transcriptional regulation. Molecular analyses were carried out to confirm the mutagenesis, followed by biochemical approaches to reveal putative protein interaction partners or RNA targets.

Given that all known members of the OPR family investigated so far in *C. reinhardtii* are involved in chloroplast gene expression it became particularly interesting to elucidate the function of the single OPR protein, AtRAP, encoded by the nuclear genome of *A. thaliana*. To gain insights, mutant lines with T-DNA insertions in *AtRAP* were used for phenotypical and molecular characterization of the protein function including the investigation of putative RNA targets.

2 MATERIALS AND METHODS

2.1 Materials

All the chemicals used in this study were p.A. quality and purchased from Roth, Sigma, Merck or AppliChem if not indicated otherwise. The instruments used in this study are mentioned in the text. An overview of suppliers can be found in Table 2.1.

Table 2.1 List of all suppliers for chemicals, enzymes and laboratory equipment

Supplier	Address
Agrisera	Agrisera AB, Vännäs, Sweden
Amersham Biosciences	Amersham Biosciences Europe GmbH, Freiburg, Germany
Biometra	Biometra GmbH, Göttingen, Germany
Biozym	Biozym Diagnostik GmbH, Hameln, Germany
BioRad	Bio-Rad Laboratories, München, Germany
Epicentre	Epicentre biotechnology, Madison, USA
Fermentas	Fermentas GmbH, St. Leon-Rot, Germany
Fuji	FUJI FILM Europe, Düsseldorf, Germany
GE Healthcare	GE Healthcare, München, Germany
Hartmann Analytic	Hartmann Analytic GmbH, Braunschweig, Germany
Invitrogen	Invitrogen GmbH, Karlsruhe, Germany
Metabion	Metabion international AG, Martinsried, Germany
Miltenyi Biotec	Miltenyi Biotec, Bergisch Gladbach, Germany
Millipore	Millipore Corp., Bedford, USA
MWG Biotech	Eurofins MWG operon, Ebersberg, Germany
PeqLab	PeqLab biotechnologie, Erlangen, Germany
Photon Systems Instruments	Photon Systems Instruments, Högrova, Czech Republic
Pierce	Pierce, Rockford, USA
Promega	Promega Corporation, Madison, USA
Qiagen	Qiagen, Hilden, Germany
Roche	Roche Diagnostics GmbH, Mannheim, Germany
Roth	C. Roth GmbH & Co, Karlsruhe, Germany
Scotts	Scotts Deutschland GMBH, Hildesheim, Germany
Serva	Serva Feinbiochemika, Heidelberg, Germany
Sigma	Sigma Chemical Company, St. Louis, USA
Stratagene	Stratagene, La Jolla, USA
Thermo Scientific	Thermo Scientific, Rockfold, USA
Whatman	Whatman Paper, Maidstone, England
Zeiss	Carl Zeiss MicroImaging GmbH, Göttingen, Germany

2.1.1 Enzymes and Kits

Enzymes used for cloning were obtained from Fermentas and NEB with specific buffer systems supplied by the corresponding companies. Protease Inhibitor cocktail (PIC) was purchased from Roche and RNase A from Roth.
The following kits were used in this study according to manufacturer's protocols:

- Perfectprep Gel Cleanup Kit (Eppendorf)
- TripleMaster PCR System (Eppendorf)
- CloneJET PCR Cloning Kit (Fermentas)
- Plasmid Mini and Midi Kits (Qiagen)
- DNeasy Plant Mini Kit (Qiagen)
- TriReagent (Sigma)
- Monster ScriptTM III Reverse Transcriptase (Invitrogen)

2.1.2 Membranes

Nitrocellulose membranes were obtained from AppliChem and positively-charged Nylon membranes from Roth.

2.1.3 Antibodies

Table 2.2 List of antibodies and respective titers used in this research work

Antibody	Titer	Reference
Primary (for *C. reinhardtii*)		
Anti-RbcL	1:5000	provided by G. F. Wildner (Ruhr Universität Bochum)
Anti-PsaA	1:1000	Agrisera
Anti-D2 (Rabbit 6442)	1:2000	Biogenes
Anti-Cytb_6	1:1000	Agrisera
Anti-CP43	1:1000	Agrisera
Primary (for *A. thaliana*)		
Anti-D1 (Rabbit 1698)	1:1000	Biogenes
Anti-D2 (Rabbit 6442)	1:2000	Biogenes
Anti-LHCII	1:3000	Agrisera
Anti-PsaA	1:1000	Agrisera
Anti-RbcL	1:5000	provided by G. F. Wildner (Ruhr Universität Bochum)
Secondary		
anti-rabbit IgG HRP	1:10000	GE Healthcare
Anti-digoxigenin	1:20000	Roche

2.1.4 Oligonucleotides

All oligonucleotides were ordered from Metabion. Lyophilized oligonucleotides were resuspended in sterile, ddH_2O to a final concentration of 100 µM and stored at -20 ℃. Sequences of used oligonucleotides are denoted in respective chapters in Methods.

2.1.5 DNA-Vectors

DNA-vectors used in this work are listed in Table 2.3.

Table 2.3 List of DNA-vectors used

Plasmid	Description	Reference
pJET1.2/blunt	Cloning vector; confers ampicillin resistance in *E. coli*	Fermentas
pGEX4T-1	Overexpression vector for GST based recombinant fusion proteins under control of *lac* promoter; confers ampicillin resistance in *E. coli*	GE Healthcare
pBC1-CrGFP	pBC1 expression vector containing the *C. reinhardtii* codon adapted GFP coding sequence (CrGFP) under control of the *PsaD* 5' and 3' UTRs; confers paromomycin resistance in *C. reinhardtii* by expression of the *APHVIII* gene and ampicillin resistance in *E. coli*	Neupert et al., 2009
pBC1	pBC1 containing the *Streptomyces* aminoglycoside 3'-phosphotransferase typeVIII encoding gene (*aphVIII*) under control of *RBCS* promoter was used for the generation of PSII mutants described in this study	Sizova et al., 1996
pMS188	pMS188 containing the zeocin resistance gene (*ble*) under control of both *HSP70A and RBCS2* promoters was used for the generation of PSII mutants described in this study	Schroda et al., 2002

2.1.6 *Escherichia coli* strains

Recombinant plasmids were propagated in *Escherichia coli* (*E. coli*) strain XL1-Blue [*endA1 gyrA96 hsdR17 lac recA1 relA1 supE44 thi-1 F ́proAB lacIq Z_M15 Tn10* (*Tetr*)] (Stratagene).

2.1.7 *Arabidopsis thaliana* strains

Wild-type: *Arabidopsis thaliana* ecotype *Columbia-0* (Col-0).

Insertion mutant lines carrying T-DNA insertions were identified by searching the insertion flanking database SIGNAL (http://signal.salk.edu/cgi-bin/tdnaexpress). The *AtRAP-1* (sail_1223_C10) and the *AtRAP-2* (sail_1225_B10) mutants derive from the Syngenta Arabidopsis Insertion Library (SAIL) T-DNA collection with both of them in the *Columbia-0*

(Col-0) background (Sessions et al., 2002). The T-DNA lines were ordered from the Arabidopsis Biological Resource Center (ABRC). Construct used for generation of T-DNA lines was pDAP101 (http://www.arabidopsis.org/abrc/pDAP101.pdf).

The primers used in section 3.5.2 for detecting homozygous mutants are listed below:

31890-fw2 (P1): ACTCTCTGTTAAAAATCACAGCA

31890-fw (P2): TTAAGGGTCAAGAGATTGCTC

31890-rev (P3): AATCAAGCCCTGTACTTATAAGAA

2.1.8 *Chlamydomonas reinhardtii* strains

The *C. reinhardtii* strains used in this research work are stated in the following Table 2.4

Table 2.4 List of *C. reinhardtii* strains

Strain	**Description**	**Reference**
cc406	Cell wall deficient (*cw15)* wild-type strain	Genetic Centre, Duke University, Durham, North Carolina; Davies DR, Plaskitt A, 1971
Jex4	wild-type mt^+ strain with cell wall	Houille-Vernes et al., 2011
XS1	cw15 arg7 mt^+	Johnson, 2007
Fud7	Deletion mutation spanning the *psbA* gene in CC-741	Bennoun et al., 1980
nac2-26	Cell wall deficient (*cw15*) Photosystem II mutant	Boudreau et al., 2000
222E	A nuclear mutant specifically fails to accumulate *psbB* transcripts	Monod et al., 1992
101a	PSII mutants generated by nuclear transformation of the wild-type strain Jex4 with the vector pBC1 (section 3.1)	generated by Dr. Xenie Johnson, CNRS/Université Pierre et Marie Curie, Institut de Biologie Physico-Chimique
45a		
101b		
42b		
41a		
42d		
41b		
44d		
102a		
BC1D7	PSII mutants generated by nuclear transformation of the wild-type strain XS1 with the vector pBC1 (section 3.1)	
BC1H3		
P10B3		
AP15-2Ci		
BC1H9		
Z1G4	PSII mutants generated by nuclear transformation of the wild-type strain XS1 with the vector pMS188 (section 3.1)	
Z1D8		

2.2 Methods

2.2.1 Growth of bacterial strains

E. coli strains were grown in LB medium (1% tryptone, 1% NaCl, 0.5% yeast extract, pH 7.0) under standard conditions (Sambrook J, 2001). 1.5% agar was applied to obtain LB media plates. For selection media, proper amounts of antibiotics were added under sterile conditions after the media was cooled down to approximately 60 ℃ after autoclaving.

2.2.2 Growth of *Chlamydomonas reinhardtii* strains

C. reinhardtii strains were maintained at 25 ℃ on Tris-acetate-phosphate (TAP) agar medium at medium light (30 µE m^{-2} s^{-1}) if not indicated otherwise (Harris, 2009). Liquid cultures were grown to a density of ~2 x 10^6 cells/mL in TAPS medium (TAP medium containing 1% sorbitol). For photoautotrophic growth experiments, *C. reinhardtii* strains were also cultured in high salt (HSM) medium (Sager, 1953)

2.2.3 Growth of *Arabidopsis thaliana* plants

A. thaliana seeds were stratified for 2 days at 2-5 ℃ in the dark to break dormancy and then sown out on plastic trays with soil. Plants were grown under controlled green house conditions (PFD 70-90 µE m^{-2} s^{-1}, 16 h light/ 8 h dark cycles). Fertilization with “Osmocote Plus” (Scotts) was performed according to manufacturer’s instructions.

2.2.4 Nucleic acids methods

2.2.4.1 Isolation of nucleic acids

2.2.4.1.1 Isolation of plasmid DNA from *E. coli*

Isolation of plasmid DNA from *E. coli* at small scale was carried out by alkaline lysis of bacteria using standard protocols as described (Sambrook J, 2001). For large scale and pure plasmid DNA isolation, the Midi Kit (Qiagen) was used according to the manufacturer’s protocol.

2.2.4.1.2 Isolation of genomic DNA from *Chlamydomonas reinhardtii*

The genomic DNA of *C. reinhardtii* was isolated from 50 mL (~2 × 10^6 cells/mL) *C. reinhardtii* liquid culture. The culture was harvested at 1100 g, 4°C for 6 min and DNA was extracted using the DNeasy Plant Mini Kit (Qiagen) according to manufacturer's protocol.

2.2.4.1.3 Isolation of genomic DNA from *Arabidopsis thaliana*

Genomic DNA of *A. thaliana* was isolated by vortexing of leaf material (2-3 leaves from 4-week old plants) frozen in liquid nitrogen in 1.5 mL microcentrifuge tubes with metal beads for 30s, followed by addition of isolation buffer (200 mM Tris/HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). After centrifugation (16000 g, RT, 20 min) the genomic DNA was precipitated by addition of 0.8 volumes of isopropanol to the supernatant. The precipitated DNA was collected by centrifugation (2000 g, 4°C, 30 min), washed with 75% Ethanol, and finally resuspended in 50 µL ddH_2O.

2.2.4.1.4 Isolation of total cellular RNA from *Chlamydomonas reinhardtii*

15 mL liquid cultures of *C. reinhardtii* were harvested at early log phase (~1 × 10^6–2 × 10^6 cells/mL) by centrifugation at 1100 g, 4°C for 6 min and total cellular RNA was extracted by using the TRI reagent (Sigma), according to the manufacturer's instructions.

2.2.4.1.5 Isolation of total cellular RNA from *Arabidopsis thaliana*

Total RNA of *A. thaliana* from fresh tissue (4-5 leaves from 3-week old plants or 2-3 leaves from 6-week old plants) was extracted by breaking of leaf material frozen in liquid nitrogen with metal beads in 1.5 mL microcentrifuge tube. RNA was subsequently extracted from ground tissue by using TRI reagent (Sigma), according to the manufacturer's instructions.

2.2.4.1.6 Determination of nucleic acid concentrations

The quality and quantity of nucleic acids were determined optically in ethidium bromide-stained agarose gels (section 2.2.4.2). In addition, UV absorption at 260 nm was measured by a Novaspec III photometer (Amersham Biosciences), and concentrations were calculated assuming an optical density OD_{260} = 1 corresponding to 50 µg/mL for double-stranded DNA and 40 µg/mL for RNA. The purity of the sample was estimated by the ratio of absorption at 260 to 280 nm.

2.2.4.2 Nucleic acid electrophoreses

2.2.4.2.1 Agarose gel electrophoresis of DNA

DNA samples were supplemented with 6 × DNA loading buffer (0.25% (w/v) bromophenol blue, 30% (v/v) glycerol) and separated depending on their size in 1-2% (w/v) agarose (Genxpress) gels containing 0.5 µg/mL ethidium bromide in 1 × TAE buffer. Using 1 × TAE as running buffer, electrophoreses were carried out at 5-10 V/cm in a horizontal gel apparatus (i-Mupid, Advance). Following electrophoresis, DNAs were visualized under UV light employing an Alpha Imager station (Alpha Innotech Corporation). 5 µL of standard DNA ladders (Fermentas) were used as molecular weight markers.

1 × TAE: 40 mM Tris-Cl, pH 8.0
20 mM acetic acid
2 mM EDTA

2.2.4.2.2 Agarose gel electrophoresis of RNA

RNA samples were supplemented with equal volumes of 2 × RNA loading dye and denatured at 65 ℃ for 5 min prior loading to the gel. Depending on the demands of experiments, RNAs were electrophoretically separated in 1-2% formaldehyde-agarose gels (1/40 vol formaldehyde in 1 × MOPS buffer). Electrophoresis was carried out in a horizontal electrophoresis gel chamber (Life Technologies) at 80 V. Fractionated RNA were visualized under UV trans-illumination by using an Alpha Imager station (Alpha Innotech Corporation). 2 µL of high range RNA markers (Fermentas) were applied to control the length of the separated RNA fragments.

1 × MOPS buffer: 20 mM MOPS
5 mM Na-acetate
1 mM EDTA pH 7.0

2 × RNA loading dye: 50% (v/v) formamide
17.5% (v/v) formaldehyde
20% glycerol
1.25 mM EDTA, pH 8.0
1.27 mM ethidium bromide
0.2% (w/v) bromophenol blue
in 1 × MOPS buffer

2.2.4.3 cDNA synthesis and RT-PCR

For cDNA synthesis, reverse transcription (RT) reactions were performed with 100-500 ng of total cellular RNA by using the MonsterScript^{TM} 1ST-Strand cDNA Synthesis Kit according to the manufacturer's instructions. Prior to RT reactions, RNAs were treated with DNase I (RNase free; Promega) to avoid contaminations with genomic DNA. 0.25 µL cDNA were used for subsequent PCR reactions (section 2.2.4.4.1).

2.2.4.4 Cloning

Standard cloning procedures were performed as described in Sambrook and Russell (2001). Purification of plasmid DNAs by QIAquick Gel Extraction Kit (Qiagen), DNA ligations by means of T4 DNA ligase (Fermentas), restriction cleavage by endonuclease enzymes (Fermentas) were performed according to the manufacturer's instructions. For direct cloning of PCR products in the pJet1.2 cloning vector, the Clonejet cloning kit (Fermentas) was used according to the manufacturer's instructions.

2.2.4.4.1 Polymerase chain reaction (PCR)

Specific DNA sequences from genomic, plasmid and cDNA were amplified using Taq DNA polymerase in a Master Cycler (Eppendorf). Standard 25 µL PCR reactions were prepared with PCR buffer (670 mM Tris-Cl pH 8.0, 67 mM $MgCl_2$ and 0.01% Tween20), 200 µM dNTPs and 10 pmol of each primer. The amount of DNA template varied from 1 ng (plasmid DNA) to 100 ng (genomic DNA). After a 3 min denaturation step at 94 ℃, 35 cycles were performed, which included denaturation at 94 ℃ (1 min), annealing at 50-65 ℃ (45 seconds), depending on Tm of primer set, and extension at 72 ℃ (1 min/kb). An additional elongation step was carried out at 72 ℃ for 10 min. PCR products were analyzed by agarose gel electrophoresis (section 2.2.4.2.1).

For synthesis of digoxigenin labeled probes used in section 2.2.4.6 (Southern) and section 2.2.4.7 (Northern), dNTPs were replaced by dig-dNTP. The mixture of dig-dNTP was set up as following: dATP (2 mM), dCTP (2 mM), dGTP (2 mM), dTTP (1.3 mM), dig DIG-11-dUTP (Roche) (0.7 mM).

2.2.4.4.2 Sequencing

For sequencing of plasmids, samples containing 200–300 ng of DNA with 10 pmol of the respective primer listed in Table 2.5 were prepared. The sequencing of DNA samples was carried out by the department of Genetics LMU.

Table 2.5 Primers used for sequencing

Name of primers	Sequence 5' to 3'
inv-APHV-rev2 (P4)	TTGATTTTGGCCTCTTTCTCCATGG
PsaD FW (for pBC1; *ohp2* complementation)	AGGTTTCCTCGCCGAGCAAG
PsaD RV (for pBC1; *ohp2* complementation)	TCCGATCCCGTATCAATCAG
pJET1.2 Forward	CGACTCACTATAGGGAGAGCGGC
pJET1.2 Reverse	AAGAACATCGATTTTCCATGGCAG

2.2.4.4.3 Transformation of *E. coli*

For heat shock transformation, plasmid DNAs or ligation products were added to 50 µL aliquots of competent XL1 blue cells and incubated on ice for 5 min. After the incubation, the cells were transferred to 42 ℃ for 45-60 seconds and then immediately cooled on ice for 2 min. The transformants were plated on selective LB agar plates and incubated 14 to 16 h at 37 ℃.

2.2.4.5 Inverse PCR on *Chlamydomonas reinhardtii* genomic DNA

Genomic DNA of *C. reinhardtii* mutants was isolated as described in section 2.2.4.1.2. 100 ng gDNA was digested by restriction enzymes *Pst*I or *Nhe*I (with none and one cutting site in pBC1 cassette, respectively). Digestion products were purified by the phenol/chloroform method (Sambrook J, 2001), followed by NaAc-ETOH precipitation, and finally resuspended in 20 µL ddH_2O. To obtain circular DNA templates for subsequent PCR reactions, digestion products were self-ligated by T4 DNA Ligase in 100 µL reaction systems over night at 16℃. Ligation products were purified as described above and used as templates for nested PCR reactions. The primary PCR was performed with first set of primers (P1 and P2) using 5 µL ligation products as template. The resulting product was then used as template for a second PCR reaction using another set of primers: P3 and P4 (for positions of primers compare Figure 3.2.3 in results section).

The PCR reactions were carried out as described in chapter 2.2.4.4.1, with primers shown in Table 2.6. The obtained product was sequenced as described in section 2.2.4.4.2 to obtain the flanking regions of mutants.

Table 2.6 Primers used for inverse PCR

Name of primers	Sequence 5' to 3'
inv-APHV-fw (first set of primers P1)	GTCCATGCTTCGAAATTCTTCAGC
inv-APHV-rev (first set of primers P2)	TGTCCGTTCGATCGCAGTCT
inv-APHV-fw2 (Second set of primers P3)	GTTGCAAGTCAAATCTGCAAGCAC
inv-APHV-rev2 (Second set of primers P4)	TTGATTTTGGCCTCTTTCTCCATGG

2.2.4.6 Southern blot (digoxigenin labeled DNA probes)

C. reinhardtii genomic DNA was prepared using the Plant DNA miniprep kit as described in section 2.2.4.1.2. The procedures for Southern blot were performed generally as described by Sambrook and Russell (2001). 10 µg gDNA were digested with appropriate restriction enzymes overnight in 100 µL reaction systems. Digestion products were precipitated by NaAc-ETOH method, and resuspended in 25 µL ddH_2O. Electrophoresis was performed in 0.8% Agarose gels at 40 V in 1 × TPE (360 mM Tris, 300 mM Na_2HPO_4, 70 mM EDTA) overnight. Agarose gels were stained with EB (0.2 pg/mL) for 20 min in 1 × TPE buffer and photographed under UV light beside a fluorescent ruler. The gel was depurinated in 0.25 M HCl for 15 min, followed by incubation in denaturing solution (0.4 M NaOH, 0.6 M NaCl) for 30 min, and neutralization solution (1 M Tris-HCl pH 8.0, 1.5 M NaCl) for 30 min. After briefly soaking the gel in 20 × SSC (3 M NaCl, 0.3 Na_3Citrate, pH 7.0), DNAs were transferred to Roti Nylon$^+$ membranes (Roth) for 15 h. The blot was washed in 2 × SSC, and then cross-linked by UV light (UV Crosslinker, UVC 500, Hoefer). After 1 h prehybridization at 68 ℃ with prewarmed pre-Hyb solution (0.25M Na_2HPO_4, pH 7.2, 1 mM EDTA, 20% SDS, 0.5% Block reagent (Roche)), 2.5 ng/mL pre-Hyb buffer of a gene specific digoxigenin labeled probe (obtained by PCR as described in section 2.2.4.4.1 using primers denoted in Table 2.7) was added, and hybridization was performed overnight at 68 ℃. The DNA blot was washed 3 × 20 min with Hyb-wash buffer (20 mM Na_2HPO_4, 1 mM EDTA, 1% SDS) at 65 ℃, then 1 × 5 min in wash buffer I (0.1 M maleic acid, pH 8.0, 3 M NaCl, 0.3% Tween20) at room temperature. After 1 h incubation in blocking buffer (1% block reagent (Roche), 0.1 M maleic acid, 3 M NaCl, 0.3% Tween), an anti-Digoxigenin Antibody (listed in Table 2.2) was applied. After 30 min, the blot was washed 4 × 10 min with wash buffer I. For signal detection, CDP star (Roche, 1:100) was applied in substrate buffer (0.1 M Tris-HCl, 0.1 M NaCl, 50

mM $MgCl_2$) on the equalized blot, and chemiluminescence detected by exposure to X-ray films (Fuji).

Table 2.7 Primers used for Southern blot analysis

Name of primers	Sequence 5' to 3'
nac2-fw	GGATGTCCGTTGCAGTTTGTTGA
nac2-rev	CCATTCACGCCGTTGTGCTTA
101b-fw (P5)	AACCTGTACAACCAGGCTTTG
101b-rev (P6)	TGCACAACGTACAACACTCAC
OHP2-fw2 (previously named OHP1-fw2)	GTGCACACGTATCACTTCTG
OHP2-rev2 (previously named OHP1-rev2)	GCACACTTACCAGTCAGCAT
45a-fw	TAGACGCAATGGAACGCAAGT
45a-rev	TGTCTCTTTACTCCCTGACTC
42b-fw	ATGGTTCAATCATGTCGGGCAACT
42b-rev	AAGCAAACACATAGACACGCATGC
hit2-fw (for *101a* Figure 3.2.4)	ATTAACAGCACCCTGCAACTACCA
hit2-rev (for *101a* Figure 3.2.4)	AACGTGATGCTGCCGTCATAAGGT
pBC1-APHV-fw	AGACTGCGATCGAACGGACA
pBC1-APHV-rev	GCTGAAGAATTTCGAAGCATGGAC
pMS-ble-fw	CACAAAGGCTAGGCGCCAATGCAA
pMS-ble-rev	TCCATTTACACGGAGCGGGGAT

2.2.4.7 Northern blot (digoxigenin labeled probe)

Total RNA from either *C. reinhardtii* or *A. thaliana* was extracted as described in sections 2.2.4.1.4 and 2.2.4.1.5. The procedures for Northern blots were generally performed as described by Sambrook (2001). In brief, total RNA separated on denaturing formaldehyde agarose gels (1% or 2%), was transferred to Roti Nylon$^+$ membrane (Roth), followed by UV light cross-linking (UV Crosslinker, UVC 500, Hoefer). Dig-labeled probes were synthesized by PCR as described in section 2.2.4.4.1 using primers denoted in Table 2.8. Hybridizations and detection of dig-labeled probes are the same as described in section 2.2.4.6.

Table 2.8 Primers used for Northern blot analysis

Name of the primers	Sequence 5' to 3'
P1-fw (for probe A in Figure 3.4.8)	TCGCTGTGATCGAATAAGAA
P1-rev (for probe A in Figure 3.4.8)	GCTTCCTTCTTCGTAGACAA
P2-fw (for probe B in Figure 3.4.8)	AGGGAGAGCTAATGCTTCTT
P2-rev (for probe B in Figure 3.4.8)	AACGAAAGAAGGCTTCCACC
P3-fw (for probe C in Figure 3.4.8)	GGGCTATTAGCTCAGTGGTA
P3-rev (for probe C in Figure 3.4.8)	GGGCGCGCTCTACCACTGAG

4.5Srrn-fw	CGAGACGAGCCGTTTATCAT
4.5Srrn-rev	TTCAAGTCTACCGGTCTGTT
5Srrn-fw	ATTCTGGTGTCCTAGGCGTA
5Srrn-rev	ATCCTGGCGTCGAGCTATTT
23Srrn-fw	TTCAAACGAGGAAAGGCTTA
23Srrn-rev	AGGAGAGCACTCATCTTG
atpA-fw	CTTAGGTCGTGTAGTTGACG
atpA-rev	CTGCAGAACCTACACGTGAT
cemA-fw	ATTTATTTTCCTGACGGCGA
cemA-rev	TACCTGTTTGACTTTCTGGA
PsbK-fw	ATGACAACTTTAGCACTGGTACTT
PsbK-rev	TTAACGGAAACTAACAGCTGCTTG
PsbM-fw	TTAGTCTTGAGTAGAAGCTGTTTT
PsbM-rev	ATGGAAGTAAATATTTACGGATTA
PsbZ-fw	AAACTACAAATGAGTTTAAAATAC
PsbZ-rev	ATGACATCAATCCTTCAAGTT
PsbT-fw	TTATTTAATCATACGTGGAGGAT
PsbT-rev	ATGGAAGCTTTAGTATATACTTT
PsbL-fw	ATGGCTAGACCAAATCCAAAT A
PsbL-rev	TAGTTAAAGATATAACTAGA
PsbI-fw	ATGTTAACATTAAAAATTTTTGTT
PsbI-rev	TTAGTCTAAATTTTTACCTGG
cemA3'-atpH Fw	TACAACCAAATAGGTTTCAATAG
cemA3'-atpH Rev	CCATACCAGGACCAATAGC
atpA5’ Fw	GCCACTGTTCACTCCTC
atpA5’ Rv	TCTGGAGTACGCATTGCC
rbcL Fw	AAGATTCAGCAGCTACAGC
rbcL Rv	CACTGCCTCTAATAAAGTCTAC
psbD Fw	GCCGTAGGGTTG AATG
psbD Rv	GTTGGTGTCAACTTGGTGG
psbA Fw	TCTAGCCTATGGGCTCGT
psbA Rv	ACCGAAACGGTAACCTTC

2.2.5 Protein methods

2.2.5.1 Determination of protein concentrations

Protein concentrations were determined as described by Bradford (1976), using the Roti®-Quant protein assay (Roth).

2.2.5.2 Total protein preparation from *Chlamydomonas reinhardtii*

Total protein from cell wall less *C. reinhardtii* strains was prepared from 15 mL light green culture growing in dim light. Cells were collected by centrifugation for 5 min, 1000 g, at room temperature. 200 µL-400 µL 2 × lysis buffers (120 mM KCl, 20 mM tricine pH 7.8, 0.4 mM EDTA, 5 mM β-mercaptoethanol, 0.2% -1% Triton ×100) was used for resuspension. Lysis was performed by mixing thoroughly with pipette on ice. The protein concentration was measured by Bradford Protein Assay (Bradford et al., 1976).

Total protein from *C. reinhardtii* with cell wall was prepared from 15 mL light green culture grown in moderate light (30 µE m^{-2} s^{-1}). Cells were collected by centrifugation for 5 min, 1000 g, at room temperature. 500 µL lysis buffer (200 mM Tris-Cl, pH 8.0, 150 mM NaCl, 50 mM $MgCl_2$, 20 mM EDTA, 0.02 g/mL Nonidet P40, 5 mM β-mercaptoethanol) was used for resuspension. 1/2 volume of glass beads (0.5 mm dia) were added into resuspension, followed by vortexing for 1 min to break the cell wall. The supernatant collected by centrifugation for 5 min at 20000 g, 4 ℃ was used for further immunoblot assays (section 2.2.5.7).

2.2.5.3 Total protein preparation from *Arabidopsis thaliana*

Total protein from *A. thaliana* was obtained from 3~8-week-old leaves. Leaves were frozen in liquid nitrogen, subsequently broken with metal beads by vortexing for 1 min. Solubilisation buffer (100 mM Tris pH 8.0, 50 mM EDTA pH 8.0, 0.25 M NaCl, 1 mM DTT and 0.7% SDS) was added to the ground tissue, then incubated at 68 ℃ for 10 min and centrifuged at 16000 g for 10 min at room temperature to remove cellular debris. The supernatant was used for protein analysis by SDS-PAGE and immunoblot described in sections 2.2.5.6 and 2.2.5.7, respectively. The protein concentration was measured by Bradford Protein Assay (Bradford, 1976).

2.2.5.4 Membrane protein preparation from *Chlamydomonas reinhardtii*

For *C. reinhardtii* membrane protein preparations used for BN-gels (section 2.2.5.8), cells (~2 $\times 10^6$ cells/mL) were harvested from 20 mL culture by centrifugation at 6000 g, 10 min, 4 ℃, and resuspended in 1 mL TMK buffer (10 mM Tris/HCl, pH 6.8; 10 mM $MgCl_2$; 20 mM KCl, with protease inhibitors). Cells were broken with glass beads by vortexing 2 × 60 seconds with an intermediate cooling on ice for at least 2 min, and centrifuged at 3000 g for 1 min to remove glass beads. Supernatants were collected and centrifuged for 10 min, 20000 g at 4 ℃. The pellet was washed twice with 500 µL TMK buffer, and finally resuspended in 500 µL

TMK buffer. The chlorophyll concentration was measured as follows: 20 µL resuspended membranes were added to 980 µL 80% acetone, incubated for 30 min in the dark, and centrifuged for 10 min, 2800 g at 4 ℃. The supernatant was collected to measure the OD_{652} in a spectrophotometer (Bio Rad). Chl a (mg/ml) = $A_{652\ nm}$ x 1.45. The obtained membrane proteins were used for 2D blue native PAGE analyses (section 2.2.5.8).

2.2.5.5 Chloroplast isolation and thylakoid extraction from *Arabidopsis thaliana*

Leaves of 3~8-week-old plants were homogenized in homogenization buffer (0.45 M Sorbitol, 20 mM Tricine-KOH pH 8.4, 10 mM EDTA, 10 mM $NaHCO_3$ and 0.1% BSA). The mixture was filtered through a double-layer-Miracloth (Calbiochem), and the filtrate was centrifuged at 300 g, 4 ℃ for 4 min in a Beckman JA-14 rotor. The pellet was resuspended carefully in resuspension buffer (0.3 M Sorbitol, 20 mM Tricine-KOH pH 8.4, 2.5 mM EDTA and 5 mM $MgCl_2$), and the suspension was centrifuged (low acceleration, no break) through a 2-step-Percoll gradient (40%-80% (v/v) in 1 × resuspension buffer) in a JS13-1 rotor, at 3250 g, 4 ℃ for 20 min. Intact chloroplasts were harvested at the interface of the 2-step-percoll gradient, washed once with resuspension buffer and centrifuged in a JS13-1 rotor at 1000 g, 4 ℃ for 4 min.

For thylakoid extraction, intact chloroplasts were lysed in extraction buffer (30 mM HEPES-KOH pH 8.0, 60 mM KOAc and 10 mM MgOAc) by passing the suspension through a 24-gauge syringe 50 times, and centrifuged at 4 ℃, 16000 g for 60 min. The pellet (thylakoid membrane fraction) was washed twice with TMK buffer (10 mM pH 6.8 Tris-HCl, 10 mM $MgCl_2$ and 20 mM KCl) and used for further analysis by 2D BN-gels (section 2.2.5.8).

2.2.5.6 SDS polyacrylamide gel electrophoresis (SDS PAGE)

The concentration of protein obtained in section 2.2.5.2 and 2.2.5.3 was determined by Bradford Protein Assay (Bradford, 1976).

Identical amounts of proteins were separated in discontinuous polyacrylamide gels according to their molecular weight as described by Laemmli (1970). A BioRad miniProtein II gel system (BioRad) was used to prepare and run the gels. Protein samples were solubilized in 5 × SDS loading buffer (10% SDS, 20% glycerol, 20% ß-mercaptoethanol and 0.1% bromophenol blue in 250 mM Tris-Cl pH 6.8), denatured at 95 ℃ (soluble proteins) or 55 ℃ (membrane proteins) for 10 min, loaded and separated by SDS-PAGE (10% -16% acrylamide) as described by Schagger and von Jagow (1987). The running buffer used for gel electrophoresis consisted of 25 mM Tris-Cl, 0.192 M glycine and 0.1% SDS. After

electrophoresis, proteins were visualized by Coomassie Blue stained gels, or detected by using immunoblot analyses (section 2.2.5.7).

2.2.5.7 Immunoblot assays

Proteins were separated by SDS-PAGE (section 2.2.5.6), transferred to nitrocellulose membranes (pore size 0.45 µm, AppliChem) in transfer buffer (48 mM Tris, 39 mM glycine, 0.037 % SDS, 20% MeOH) for 1.5 h at 0.8 mA/cm^2 using a semi-dry blot transfer apparatus (Peqlab). To visualize the quality of transferred protein, the membrane was stained briefly in Ponceau S solution (0.2% Ponceau S, 1% acetic acid). Subsequently, the nitrocellulose membrane was incubated in blocking buffer (5% milk powder in 1 × TBS-T (150 mM NaCl, 7.7 mM Tris-Cl pH 7.5, 0.1%Tween)) for 1-2 h at room temperature, followed by incubation with the primary antibody in blocking buffer at 4 ℃ overnight. Dilutions of respective antibodies are denoted in Table 2.2. Afterwards, the nitrocellulose membrane was washed 3 × 10 min in 1 × TBS-T, and then incubated with the secondary antibody for 1 h at room temperature. The membrane was again washed 3 × 10 min in 1×TBS-T. HRP conjugated antibodies were detected by enhanced chemiluminescence (ECL) solution (Thermo Scientific) and exposure to X-ray films (Fuji).

2.2.5.8 2D Blue Native-PAGE

For the first dimensional analysis of total membranes from *C. reinhardtii* or thylakoid membranes from *A. thaliana*, respectively, (compare sections 2.2.5.4 and 2.2.5.5) by Blue Native-PAGE, membranes corresponding to 25 µg of chlorophyll were resuspended in 60 µL ACA Buffer (750 mM ε-aminocaproic acid, 50 mM Bis-Tris-Cl pH 7.0, 5 mM pH 7.0 EDTA, 50 mM NaCl). Thylakoids were solubilized on ice for 10 min by applying n-dodecyl-β-D-maltoside (β-DM) to a final concentration of 1.5% (w/v). After centrifugation at 16000 g, 4 ℃ for 20 min, the supernatant was mixed with 1/20 volume of BN loading dye (750 mM ε-aminocaproic acid and 5% Coomassie G 250 (w/v)) and fractionated in a non-denaturing 4.5%-12% BN-PAGE gel (polyacrylamide gel containing 0.5 M ε-aminocaproic acid, 20% glycerol and 50 mM Bis-Tris-Cl pH 7.0). The BN-PAGE was carried out overnight with cathode buffer (50 mM Tricine, 15 mM Bis-Tris-Cl pH 7.0 and 0.02 g/ mL Coomassie G 250) and anode buffer (50 mM Bis-Tris-HCl pH 7.0) at a constant voltage of 70 V at 4 ℃. A Biometra maxigel system (G 48) was used to run the gels.

For the second dimension (2D-PAGE), gel lanes from the first dimension were cut off, denatured in denaturing buffer (0.125 M Tris-HCl pH 6.8, 4 g/mL SDS and 1% β-

mercaptoethanol) for 30 min at room temperature, and resolved on the second dimension of Urea gel (15% acrylamide, 60 g/ mL urea). Gels were either stained with Coomassie Blue/ silver staining to visualize proteins or specific proteins were detected using immunoblot detection (section 2.2.5.7).

2.2.5.9 *In vivo* translation assay of *Chlamydomonas reinhardtii* thylakoid proteins

In vivo radioactive ^{35}S labeling of *C. reinhardtii* thylakoid proteins was principally performed as described by Klinkert (2006). 50-100 mL *C. reinhardtii* culture with a density of 2×10^6 cells/mL were collected by centrifugation at 1000 g, 5 min at room temperature, washed carefully once with TAPS-B buffer and then resuspended in 10 mL TAPS-B buffer (composition described below). The cultures were transferred to sterile 50 mL Erlenmeyer flasks. After shaking for 16 h in medium light, cells were collected again by centrifugation (1000 g, 5 min, RT), washed carefully in TAPS-B/T buffer (composition described below), and finally resuspended exactly in 10 mL TAPS-B/T. The cultures were shaken for 2 h in the dark for sulfur-starvation. The chlorophyll content of the cells was measured by acetone assay as described in section 2.2.5.4. and resuspended to a final density of 80 µg chl/mL. 250 µL of this suspension were incubated with 25 µL cycloheximide stock solution (100 µg/mL) in the dark for 10 min. 12.5 µL ^{35}S (10 mCi/mL, Hartmann analytic) was added to the culture, immediately followed by illumination with light (50 µE/m^2/s) for 30 min. The labeled cells were harvested by centrifugation at 20000 g for 15 min, broken by pipetting in lysis buffer A (10 mM HEPES pH 7.8, 10 mM EDTA, protease inhibitor cocktail). Membranes were collected by spinning down at 20000 g for 25 min, 4 ℃, and resuspended in 100 µL buffer B (10 mM Tricine, 10 mM EDTA, pH 7.8).

Membrane proteins were resolved in denaturing 16% SDS PAGE gel with 60 g/ mL urea. The gel was stained with Coomassie Blue to visualize proteins according to standard protocols, and dried on a Whatman filter (MUNKTELL) by a dryer (Model 583, Bio Rad). After exposure to a PhosphoImager Screen, radioactive labeled proteins were detected and quantified with the Typhoon PhosphorImager (GE healthcare).

- TAPS-B (1 L): Tris base 2.42 g
  0.5 mM KPO_4
  100 × Beijerink Pulse 10 mL
  Trace 1 mL
  Sorbitol 10 g, pH 7.0

- TAPS-B/T (1 L): Tris base 2.42 g

0.5 mM KPO_4
100 × Beijerink Pulse 10 mL
Sorbitol 10 g, pH 7.0

- 100 × Beijerink Pulse(1 L): NH_4Cl 40 g
$CaCl_2 \times 2\ H_2O$ 5 g,
$MgCl_2 \times 6\ H_2O$ 8.25 g

- Trace (1 L): $FeSO_4 \times 7H_2O$ 4.99 g
Na_2EDTA 50 g
$ZnSO_4 \times 7H_2O$ 22 g
H_3BO_3 11.4 g
$MnCl_2 \times 4H_2O$ 5.06 g
$CoCl_2 \times 6H_2O$ 1.61 g
$CuSO_4 \times 5H_2O$ 1.57 g
$(NH_4)_6Mo_7O_{24} \times 4H_2O$ 1.1 g
pH 6.5-6.8

2.2.5.10 *In vivo* translation assay of *Arabidopsis thaliana* thylakoid proteins

In vivo radioactive ^{35}S labeling of thylakoid proteins was performed as described by Pesaresi (2006). Five *A. thaliana* leaves harvested at the 12-leaf rosette stage were pressed softly against a sandpaper, and vacuum infiltrated in a 20 mL syringe with 5 mL TME Buffer (20 mM Tris-Cl pH 6.8, 10 mM $MgCl_2$ and 5 mM EDTA) containing 1 mCi ^{35}S Methionine, 20 µg/mL cycloheximide and 0.1% Tween-20. After infiltration, leaves were illuminated with light (50 µE m^{-2} s^{-1}) for 30 min. Thylakoids were isolated and resolved on SDS-PAGE as described in section 2.2.5.5 and 2.2.5.6. The gel was stained with Coomassie Blue, and dried on a Whatman paper (MUNKTELL) by the drier (Bio Rad). After exposure to a PhosphorImager screen, radioactive labeled proteins were detected and quantified with a Typhoon PhosphorImager (GE healthcare).

2.2.6 Chlorophyll fluorescence Q_Y-max measurement

The maximum quantum yield of PSII photochemistry (Q_Y-max) was detected using the Fluorcam system (Photon systems instruments) according to the manual. *C. reinhardtii* strains were dark adapted for 10 min and minimal fluorescence (Fo) was measured. Then, pulses (0.8 s) of white light (5000 mmol photons m22 s21) were applied to determine the maximum fluorescence (Fm) and the ratio (Fm – Fo)/Fm = Fv/Fm (maximum quantum yield of PSII) was calculated.

2.2.7 Electroporation of *Chlamydomonas reinhardtii*

Nuclear transformation of cell-walled *C. reinhardtii* cells was carried out using the electroporation method (Shimogawara et al., 1998). Briefly, cells of *C. reinhardtii* were grown in 100 mL TAPS medium to a cell density of 1-2 $\times 10^6$ cells/mL. Cells were harvested by centrifugation (1100 g, 6 min, RT) and resuspended in TAPS medium to a density of 1 $\times 10^8$ cells/mL. 300 µL cells, 2 µg plasmid DNA and 40 µg carrier DNA (salmon fish sperm) were mixed in a sterile electroporation cuvette, and incubated at 16 ℃ for 20 min. In parallel, identical set-ups without plasmid DNA were used as negative controls. The electroporation was performed with standard setting (R = high range; electric field = 1 kV; C = 10 µF). After the transformation, the cuvettes with cells were incubated again at 16 ℃ for 20 min.

Selection of complemented strains was carried out by plating transformants on HSM plates, growing under high light condition for 10 days. Selection of paromomycin resistant clones was approved by diluting 250 µL of transformed cells with 25 mL TAPS medium and growing under low light for 18 h for regeneration. Afterwards the cells were collected by centrifugation at 2000 g for 5 min at RT, and then resuspended in 500 µL TAPS medium. Aliquots of 50 µL were plated on TAP agar plates supplemented with 10 µg/mL paromomycin. Plates were kept under dim light or in the dark, and scored for transformants about 2 weeks after plating.

2.2.8 Complementation of *Chlamydomonas reinhardtii*

The complementation of *C. reinhardtii* mutants was performed either by using BAC (bacterial artificial chromosome) clone DNA (for mutant *45a* and *101b*) or PCR amplified cDNA (for mutant *101a*). Electroporation was employed as described in section 2.2.7.

The BAC clones named 35E19 (109 kbp, corresponding to PTQ13131.y3) and 28D19 (53 kbp, corresponding to PTQ10634) were selected for complementation of mutants *45a* and *101b* respectively, and ordered from BAC Resource Center (Clemson University). BAC DNAs were prepared by using the Midi Kit (Qiagen) according to the manufacturer's instructions. The successful complimented strains were selected by photoautotrophic growth on HSM plates.

For complementation of *101a*, the full length cDNA sequence of *OHP2* was amplified by standard PCR reactions (section 2.2.4.4.1), using primers with additional *Nde*I and *Eco*RI cutting site, shown below:

OHP1-fw4: catatgTCGATTGCTGCACTCCG

OHP1-rev4: gaattcTTAGTCCAG GTCCACGATG

The amplified sequence was then ligated into the pBC1 vector (pJR38, Neupert et al., 2009) via *Nde*I/ *Eco*RI restriction sites, thus replacing the GFP coding sequence. This construct was then transformed into *101a* mutant via electroporation. The positive transformants were selected first by growth on paromomycin TAP plates, then photoautotrophic growth on HSM plates.

2.2.9 Crossing of *Chlamydomonas reinhardtii*

The crossing of *C. reinhardtii* was performed by Dr. Olivier Vallon (CNRS, Paris, France), as described principally by Harris (1989). In brief, healthy growing parental strains with opposite mating types were transferred to TAP-N (TAP without NH_4Cl) plates and grown for 3-4 days till the cells became yellowish. Cells of both mating types were resuspended in 1 mL ddH_2O with 1% sorbitol and shaked for 1 h under dim light. The mating step was set up by mixing these two cultures in 50 mL sterilized flasks and keeping it under high light (100 $\mu E/m^2/s$) without shaking. 70 µL of mating cells were taken by pipette and dropped on TAP (3% washed agar) plates after 2 h, 3 h and 4 h. The plates with drops were dried and kept under moderate light overnight, followed by growth in the dark for 1-2 weeks for the development of zygotes. The grown zygotes were picked up and tetrads with 4 or more progenies inside were opened carefully. Released progenies were subsequently collected by self-made glass hook for further analyses.

2.2.10 Bioinformatics sources

All software tools mentioned below were applied with default parameters unless stated otherwise.

2.2.10.1 Prediction of gene models

For gene model analysis and the obtainment of EST and genome sequences, the *C. reinhardtii* Genome Browser from the U.S. Department of Energy Joint Genome Institute (DOI JGI, (http://genome.jgi-psf.org/Chlre4/Chlre4.home.html), the UCLA browser for *C. reinhardtii* 454 EST reads (http://genomes.mcdb.ucla.edu/Cre454/) and the browser of the National Center for Biotechnology Information employing the BLASTp and tBLASTn algorithms (NCBI, http://www.ncbi.nlm.nih.gov/BLAST) were used. The applied AUGUSTUS gene models (version 10.2) and gene identifiers were generated on evidence-based predictions using the v4 Chlamy genome assembly and the program AUGUSTUS (Stanke et al., 2004).

For *A. thaliana*, the browser of The Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org) was used for gene model analysis and Blast search.

2.2.10.2 Prediction of protein localization and transit peptides

The prediction of protein localizations and transit peptides was based on Target-P version 1.1, Predotar and ChloroP (Emanuelsson et al., 2000; Emanuelsson et al., 1999; Small et al., 2004)

2.2.10.3 Protein properties and repeat predictions

The ProtParam tool on the ExPASy server was used for basic protein property predictions, like theoretical molecular weight calculations (Gasteiger et al., 2003). The OPR repeats were predicted manually in NCBI by amino acid sequence alignments with the OPR consensus sequence.

3 RESULTS

3.1 Characterization of *Chlamydomonas reinhardtii* PSII mutants

To find so far unidentified proteins involved in photosynthesis and to attain a better understanding of the regulation of chloroplast gene expression in *C. reinhardtii*, a forward genetic approach was applied. 16 PSII mutants, provided by Dr. Xenie Johnson (CNRS/Université Pierre et Marie Curie, Institut de Biologie Physico-Chimique, Paris), were obtained by random insertion of the vectors pBC1 or pMS188 into the nuclear genome of *C. reinhardtii* wild-type strains Jex4 or XS1 (sections 2.1.5 and 2.1.8, Table 3.2.1). PSII-specific mutants were selected by primary phenotypical analyses based on their inability to grow photoautotrophically on minimal medium and increased chlorophyll fluorescence as determined by Q_Y-max measurements. As summarized in Table 3.2.1, none of the mutants was able to grow photoautotrophically on minimal medium, with the exception of mutant *101b*, which showed a slight growth. All mutants revealed significant lower Q_Y-max values (0.01 - 0.42) than the wild-type (~ 0.8).

In order to gain insights into the molecular basis for the mutant phenotypes, the accumulation of core proteins from photosynthetic complexes was investigated by determination of the levels of the PSII reaction center protein D2, the large subunit of RuBisCo (RbcL), Cytb_6 of the Cytochrom b_6f complex and PsaA of the PSI complex. As shown in Figure 3.1.1, the accumulation of the D2 protein was significantly reduced in these 16 PSII mutants, which is in agreement with reduced Q_Y-max values, especially in those mutants with no detectable D2, i.e., *BC1H9* (Q_Y-max=0.09), *BC1D7* (Q_Y-max=0.02), *42d* (Q_Y-max=0.09) and *101a* (Q_Y-max=0.02). Mutants, which revealed higher Q_Y-max values (above 0.3), such as *44d* (Q_Y-max=0.42), *102a* (Q_Y-max=0.33), *101b* (Q_Y-max=0.35) and *42b* (Q_Y-max=0.37), accumulated more D2 proteins than the formerly described ones. At this, a clear correlation of increased chlorophyll fluorescence and reduced D2 protein accumulation was observed. However, almost all the mutants had wild-type protein levels of subunits from other chloroplast complexes, such as RbcL, Cytb_6 and PsaA, whereas the *Z1D8* mutant is an exception, in which the RbcL protein was completely missing (Figure 3.1.1, upper panel).

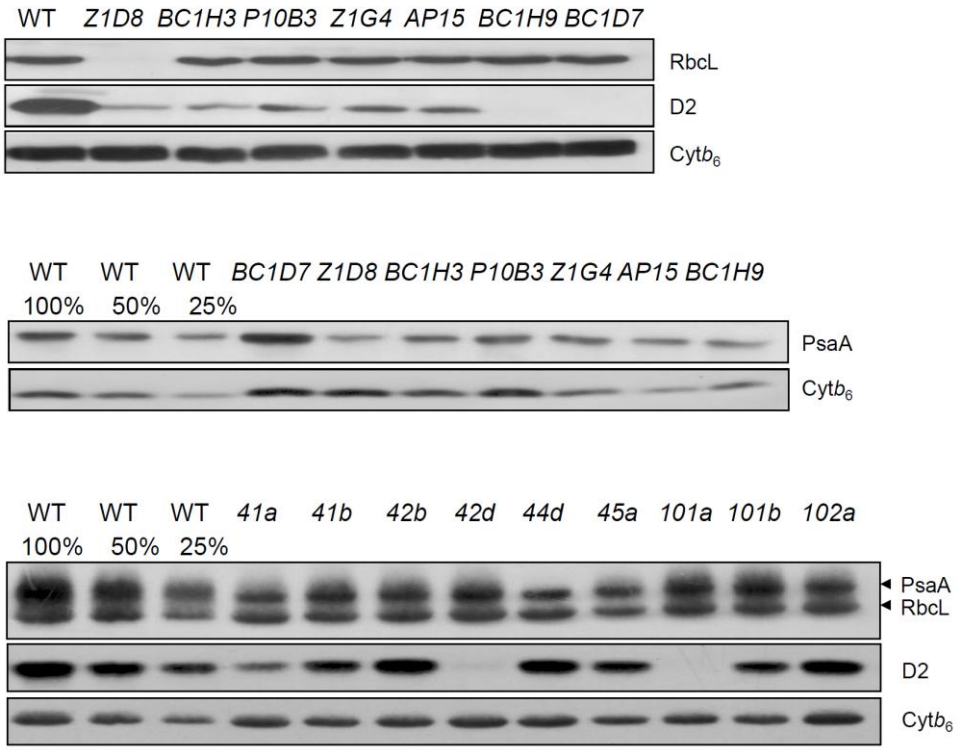


Figure 3.1.1 Accumulation of core proteins from chloroplast photosynthetic complexes in investigated PSII mutants. Immunoblots of total cell extracts from wild-type (WT) and 16 PSII mutants using antibodies against proteins indicated on the right side. Dilution series of wild-type proteins loaded in the lanes were marked as WT 100%, 50% and 25%.

In order to obtain indications if reduced transcript abundances changed protein stability or synthesis rates and were consequently responsible for reduced PSII protein levels in the described mutants, transcript accumulation analyses were performed. Northern blot analyses were accomplished by using specific probes for *psbA*, *psbD* and *rbcL*, encoding for D1, D2 and RbcL proteins, respectively (primers used for amplifying probes are listed in Table 2.8). As shown in Figure 3.1.2, almost all the mutants accumulate *psbA* and *psbD* mRNAs to wild-type levels, except *BC1D7* and *42d* mutants, which show undetectable amounts of *psbD* or *psbA* mRNA, respectively. These results indicate that except for *BC1D7* and *42d*, the reduced D2 accumulation of the rest 14 PSII mutants is due to posttranscriptional defects, as the transcript level for *psbD* is unaltered. The reason for reduced D2 accumulation can be protein synthesis or stabilization defects. These hypotheses were tested by *in vivo* labeling experiments as described below. However, the loss of *psbA* transcript in *42d* and *psbD* transcript in *BC1D7* may be due to a transcription or mRNA stabilization defect. In the *Z1D8* mutant, *rbcL* mRNA accumulates to the wild-type level, which indicates that it could be an RbcL translation or stabilization mutant.

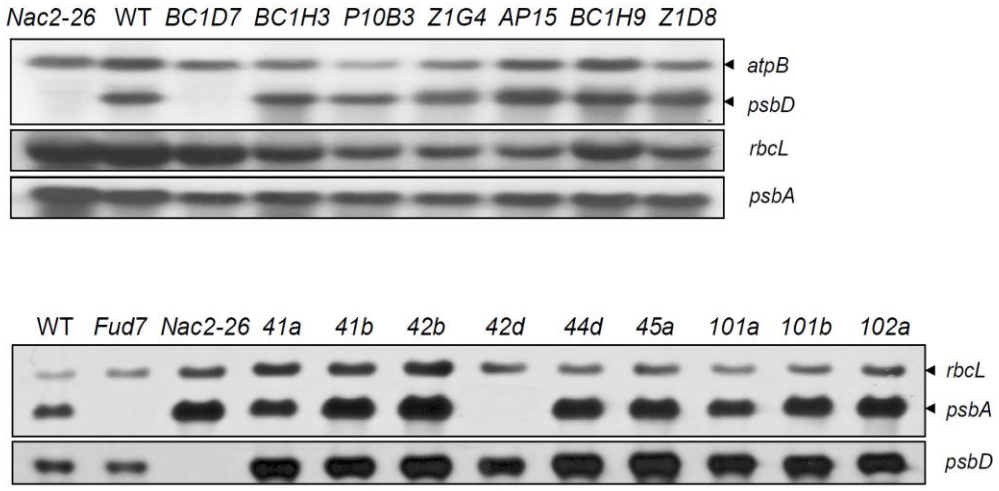


Figure 3.1.2 Accumulation of photosynthesis related chloroplast transcripts. 3 µg of total cellular RNA from wild-type and 16 PSII mutants were fractionated by denaturing agarose gel electrophoresis and blotted onto nylon membrane. Membranes were hybridized with probes specific for *psbA* and *psbD*. For loading control, the same blots were hybridized with probes specific for *atpB* and *rbcL* transcripts, respectively. The *Nac2-26* and *Fud7* mutants were employed as negative controls for *psbD* and *psbA* mRNA accumulation.

In vivo labeling of thylakoid proteins was carried out for mutants *Z1D8, Z1G4, AP15-2Ci, BC1D7, BC1H9, BC1H3, P10B3, 101a, 101b, 45a, 42b* (Figure 3.1.3). It was observed that, in *Z1G4, AP15-2Ci* and *101a* mutants, there is no detectable D1 protein synthesized. These observations suggest that these mutants are having a defect in D1 synthesis. Or alternatively, the protein is rapidly degraded. In accordance with a lack of detectable *psbD* mRNA, the *BC1D7* mutant shows no D2 synthesis. In addition, D1 is also not synthesized or rapidly degraded in the *BC1D7* mutant, while the CP47 accumulation is reduced, as expected from the CES principle (section 1.3.1.2.2). A similar phenotype has been reported previously for the *Nac2-26* mutant (Boudreau et al., 2000). Also no D2 synthesis was observed in the *BC1H9* mutant, even though no changes in *psbD* mRNA levels were observed, pointing to a D2 protein synthesis or stability defect. In *BCIH3* and *P10B3* mutants, the translation of CP43 is strongly reduced, which suggests they might share a similar molecular phenotype with the Tbc2 mutants published before (Auchincloss et al., 2002, Figure 3.1.3 A). Moreover, *45a* also showed a D2 synthesis phenotype due to undetectable D2 protein in *in vivo* labeling experiments (Figure 3.1.3 B). An additional protein accumulation was observed between the size of D1 and D2 in the *45a* mutant, which may correspond to be a precursor or modified form of the D1 protein (Figure 3.1.3 B). However, there is no clear change in thylakoid protein synthesis in *101b* and *42b* mutants, which suggests the mutated genes might encode proteins which could either stabilize the PSII subunits or are involved in the assembly of PSII complexes. Moreover, it is easy to find there is no detectable RbcL signal for *Z1D8* mutant, which is in accordance with Western blot analysis (Figure 3.1.1). According to these

observations, *Z1D8* shows multiple phenotypes, i.e. a PSII and also a RuBisCo deficient phenotype.

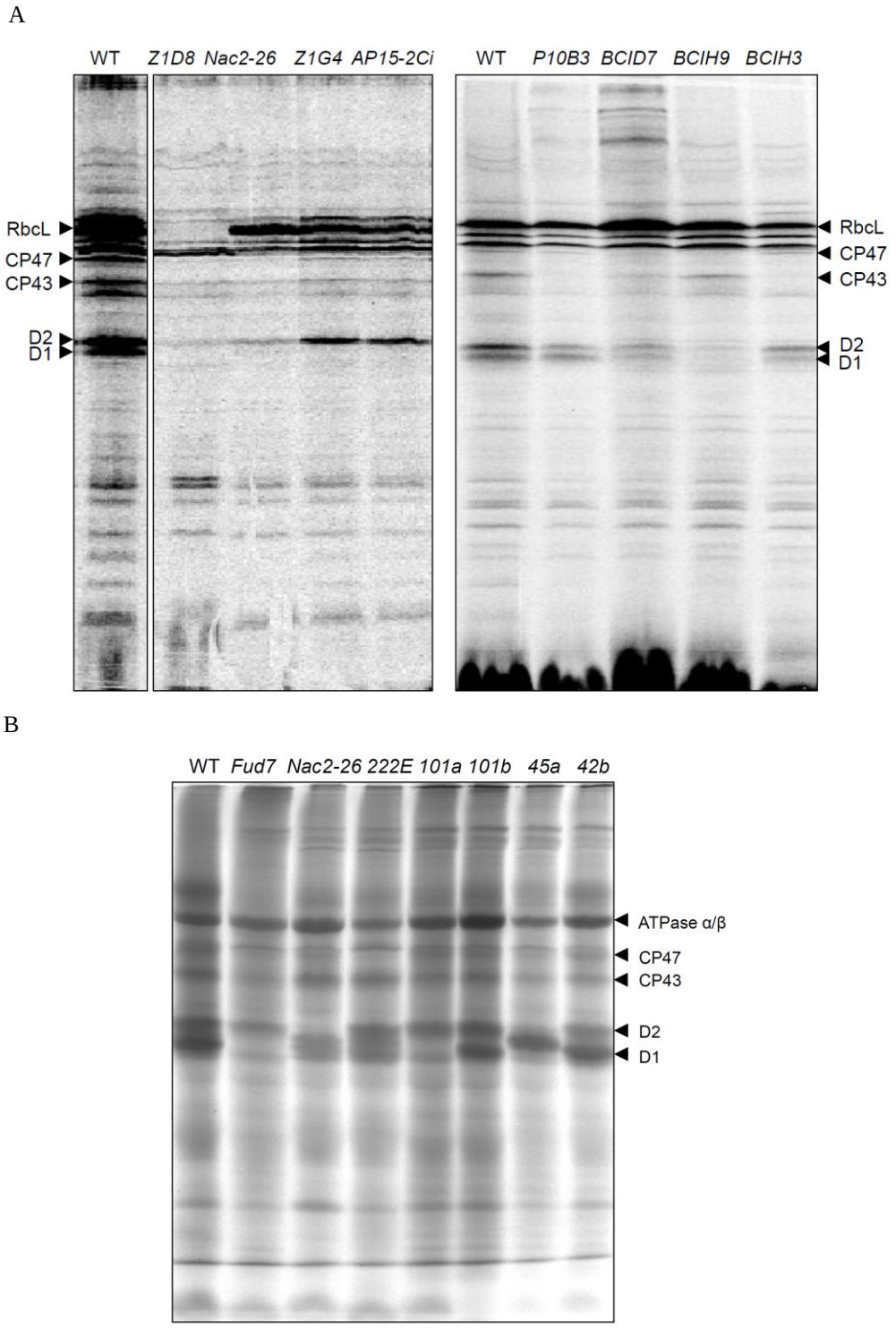


Figure 3.1.3 Synthesis of PSII subunits by radioactive *in vivo* labeling of newly synthesized proteins.
(A) Incorporation of ^{14}C into chloroplast proteins of wild-type and PSII mutants. After labeling for 20 min in the presence of cycloheximide, thylakoid membranes were isolated, fractionated by 5%~14% SDS-PAGE and detected by autoradiography (performed together by Dr. Olivier Vallon and Fei Wang).
(B) Incorporation of ^{35}S into thylakoid membrane proteins of wild-type, *Fud7* (a *psbA* mRNA deletion mutant), *Nac2-26* (a D2 protein synthesis defect mutant due to *psbD* mRNA instability), *222E* (a CP47 protein synthesis defect mutant due to *psbB* mRNA instability), and PSII mutants under investigation. After labeling for 20 min in the presence of cycloheximide, thylakoid membranes were isolated, fractionated by 14% SDS-PAGE and detected by autoradiography.

3.2 Identification of mutated genes in *Chlamydomonas reinhardtii* PSII mutants

3.2.1 Determination of copy numbers of the inserted cassette in PSII mutants

As described previously, during generation of insertional mutants, the vector is randomly integrated into the nuclear genome of *C. reinhardtii*, which can also produce mutants with multiple insertions. Since single insertional mutants are of particular interest for further molecular work, it was necessary to determine the copy number of the inserted cassette by Southern blot analysis. The probes used specifically detected either the *ble* gene on pMS188 or the *aphVIII* gene on pBC1, conferring the antibiotic resistance used for selection of *C. reinhardtii* mutants. From the Southern results, several mutants were clearly found to be single insertional mutants, which include *Z1D8*, *41a*, *41b*, *42b*, *42d*, *45a*, *44d*, *101a*, *101b* and *102a*. These mutants were certainly preferential for further molecular research. For *Z1G4*, *P10B3*, *AP152Ci* and *BC1H9*, there were at least two insertions of the respective vector found, indicating that they are multiple insertional mutants. It is worth noticing that *AP152Ci* and *BC1H9* have the same hybridization signal from both *Pst*I and *Nhe*I digestion, which supposes one of these two mutants might be contaminated by the other; further more molecular analysis is required for clarification. No visible signal was detected in *BC1D7* and *BC1H3* which might indicate the insertion of truncated transformation vectors or genomic rearrangements limiting the detection of the mutagen (Figure 3.2.1).

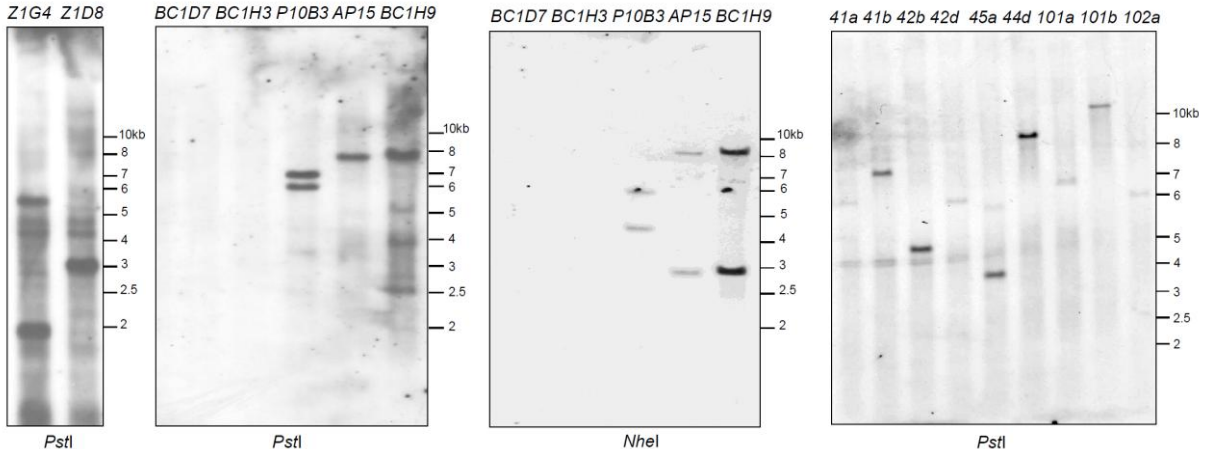


Figure 3.2.1 Southern blot analyses determining the copy number of the mutagenic vectors. 10 µg of genomic DNA were fractionated by 0.8% agarose gels after digestion by appropriate restriction enzymes labeled under each blot. Gels were blotted onto nylon membranes, and hybridized with probes specifically detecting the *ble* gene for *Z1G4*, *Z1D8* or the *aphVIII* gene for *BC1D7*, *BC1H3*, *P10B3*, *AP152Ci*, *BC1H9*, *41a*, *41b*, *42b*, *42d*, *45a*, *44d*, *101a*, *101b*, *102a* (primers used are listed in Table 2.7). Molecular size markers are labeled at the right.

Table 3.2.1 shows a detailed summary of the above mentioned results from all 16 PSII mutants under investigation.

Table 3.2.1 Summary of the phenotypical characterization of 16 investigated PSII mutants.
N.d.: not determined; 0: not detectable; WT: wild-type transcript or protein levels

Mutant	**Mutant generation**			**Photosynthetic activity**		**Protein/transcript accumulation**					**Chloroplast protein synthesis**				
	recipient strain	vector	copy number	Photoauto-trophic growth	Q_Y-max	D2 Protein	RbcL protein	*psbD* mRNA	*psbA* mRNA	*rbcL* mRNA	D1	D2	CP43	CP47	RbcL
101a	Jex4	pBC1	1	-	0.02	0	WT	WT	WT	WT	0	WT	WT	WT	WT
45a			1	-	0.24	↓					0	0	WT	↓	WT
101b			1	Slight growth	0.35	↓					WT	WT	WT	WT	WT
42b			1	-	0.37	↓					WT	WT	WT	WT	WT
41a			1	-	0.01	↓					N.d.				
42d			1	-	0.09	0			0						
41b			1	-	0.26	↓			WT						
44d			1	-	0.42	↓									
102a			1	-	0.33	↓									
BC1D7	XS1		0	-	0.02	0		0			0	0	WT	↓	WT
BC1H3			0	-	0.05	↓		WT			WT	WT	↓	WT	WT
P10B3			2	-	0.16	↓					WT	WT	↓	WT	WT
AP15-2Ci			2	-	0.23	↓					0	WT	↓	↓	WT
BC1H9			2	-	0.09	0					0	0	WT	↓	WT
Z1G4		pMS 188	2	-	0.15	↓					0	WT	WT	WT	WT
Z1D8			1	-	0.30	↓	0				0	0	↓	↓	0

3.2.2 *BC1D7* is a *Nac2* mutant

Since the PSII activity was found to be affected in all mutants described in section 3.1, a further goal was the identification of corresponding mutated genes. In order to exclude the possibility that the mutated genes are already cloned and have been published before, Southern blot analyses were carried out for all mutants with probes specifically detecting the *TBA1*, *MBB1,* and *Nac2* genes, of which the encoded proteins are known to reveal PSII related phenotypes (Boudreau et al., 2000; Somanchi et al., 2005; Vaistij et al., 2000). None of the mutants showed an insertion in these investigated genes (data not shown), with the exception of *BC1D7*. This mutant was verified to be a *Nac2* mutant, with the *Nac2* gene disrupted by an insertion (Figure 3.2.2). In addition, *BC1D7* could also be complemented with cosmid DNA (cosnac5) containing the *Nac2* gene, which further confirmed the conclusion above (performed by Dr. Olivier Vallon and Fei Wang). All of these results explain the *Nac2*-like phenotype of *BC1D7* described above (chapter 3.1), and suggest that this mutant is allelic to the previously described *Nac2-26* mutant. Hence, this mutant was excluded from further analysis.

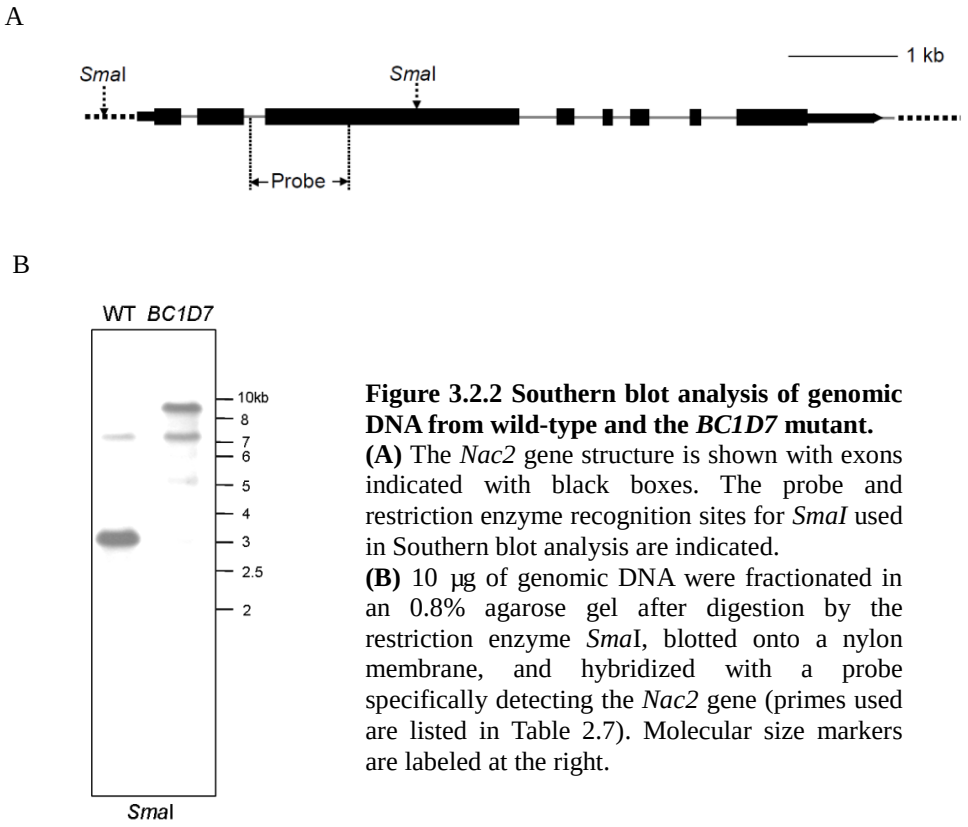


Figure 3.2.2 Southern blot analysis of genomic DNA from wild-type and the *BC1D7* mutant.
(A) The *Nac2* gene structure is shown with exons indicated with black boxes. The probe and restriction enzyme recognition sites for *SmaI* used in Southern blot analysis are indicated.
(B) 10 µg of genomic DNA were fractionated in an 0.8% agarose gel after digestion by the restriction enzyme *Sma*I, blotted onto a nylon membrane, and hybridized with a probe specifically detecting the *Nac2* gene (primes used are listed in Table 2.7). Molecular size markers are labeled at the right.

3.2.3 Identification of mutated genes by inverse PCR

Among the nine PSII mutants revealing single insertion of the mutagen, eight mutants with severely affected PSII performance (shown in chapter 3.1) were chosen for gene identification, which are *Z1D8*, *41a*, *41b*, *42b*, *42d*, *45a*, *101a* and *101b*. Therefore, the inverse PCR technique described in Figure 3.2.3.was employed.

By applying this technique, the insertional site of the mutagen was identified successfully for four of the mutants (*101b*, *45a*, *42b*, and *101a*). Products from two sets of independent PCRs with different restriction enzymes were obtained for sequencing (Figures 3.2.3 and 3.2.4; sequencing results are attached in annex). The sequencing results were analyzed by the BLAST tool in the Chlamydomonas JGI4 database. Both of the PCR products obtained by the use of two different restriction enzymes identified the same flanking genes. A hypothetic protein of 306 kDa was found to be encoded by the mutated gene in the *45a* mutant (insertion happened in the 27th exon), which revealed one Zinc finger domain and AAA-like domains, often found in helicase enzymes by a NCBI search for conserved domains. This might indicate a function of this protein in DNA repair or as a transcription factor.

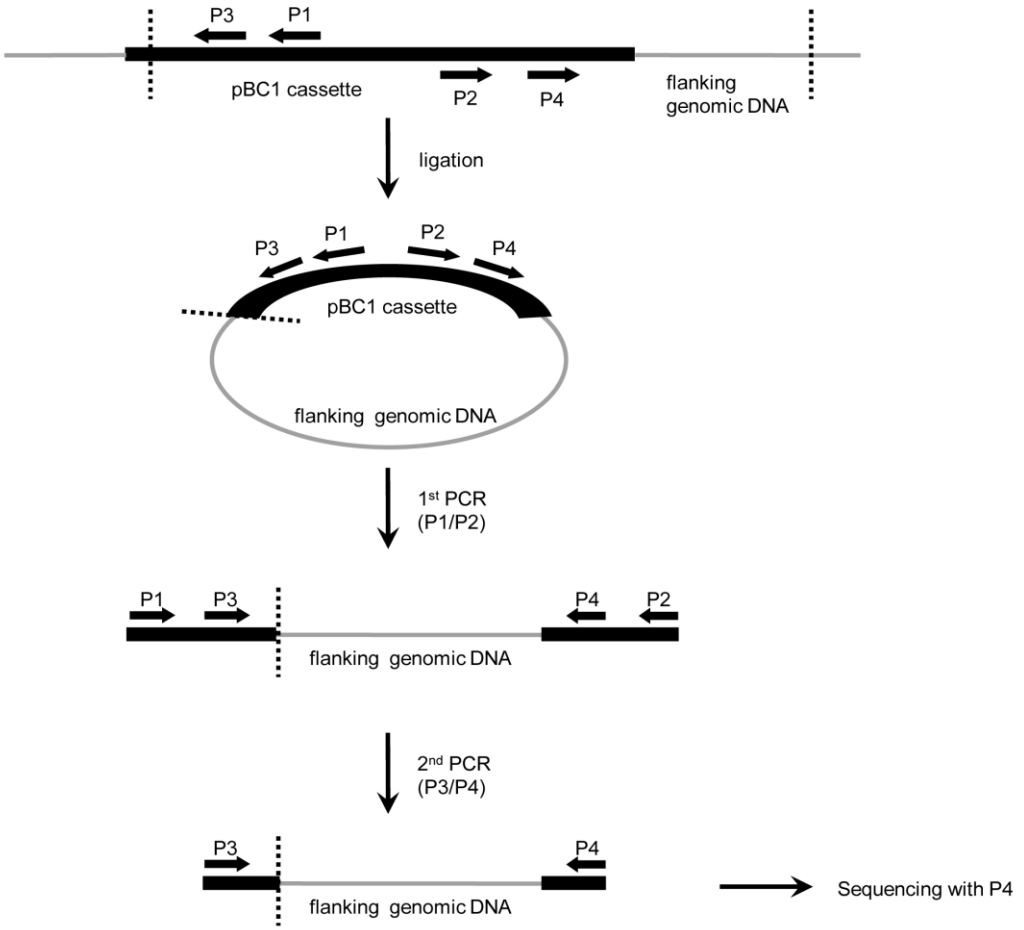


Figure 3.2.3 Inverse PCR for identification of mutated genes. The procedure is exemplarily shown for the insertion of the pBC1 vector. The mutant genomic DNA (grey line) with inserted pBC1 vector (black box) is shown, the dashed lines represent the cutting site by relevant restriction enzymes. The inverse PCR was carried out as following: the genomic DNA from PSII mutants was digested with restriction enzymes *Pst*I or *Nhe*I, respectively. The digested fragment was ligated by T4 ligase overnight to get circular DNA for inverse PCR. Primer P1 and P2 (correspond to inv-APHV-fw and inv-APHV-rev in Table 2.6) were applied for the 1st PCR, whose product was used as a template for 2nd PCR with primer P3 and P4 (correspond to inv-APHV-fw2 and inv-APHV-rev2 in Table 2.6). The PCR product was used for flanking DNA sequencing with primer P4.

Another hypothetic protein of 429 kDa was identified in the *42b* mutant (the insertion happened in the 15th exon), which has a speract/scavenger receptor domain reported for several extracellular receptors and may be involved in protein-protein interactions. However, both proteins found to be mutated in *45a* and *42b* seem to be algae specific and not conserved in the green lineage as concluded from protein Blast analysis. In *101a*, a hypothetic protein encoded on chromosome 11 was revealed (the insertion happened inside the intron between the last two exons), which contains a CUE domain reported to bind ubiquitin. In the *101b* mutant, the *CLR24* gene on chromosome 6 was found to be disrupted (insertion happened before the last exon), encoding a protein of the OPR family. Features of identified genes and gene products are summarized in Table 3.2.2.

Table 3.2.2 Summary of mutated genes in *45a, 42b, 101a,* and *101b* identified by inverse PCR

Mutant	Genome position[a]	Augustus 10 gene model[b]	Homologies[c]	Predicted protein size[d]
45a	chromosome_3:2410185-2428209	Cre03.g166850	Zn finger	306 kDa
42b	chromosome_12:8182764-8207719	Cre12.g552700	Speract/scavenger receptor domain	429 kDa
101a	chromosome_11:2188285-2191749	Cre11.g480000	CUE	31 kDa
101b	chromosome_6:2889693-2895667	Cre06.g272450	OPR	138 kDa

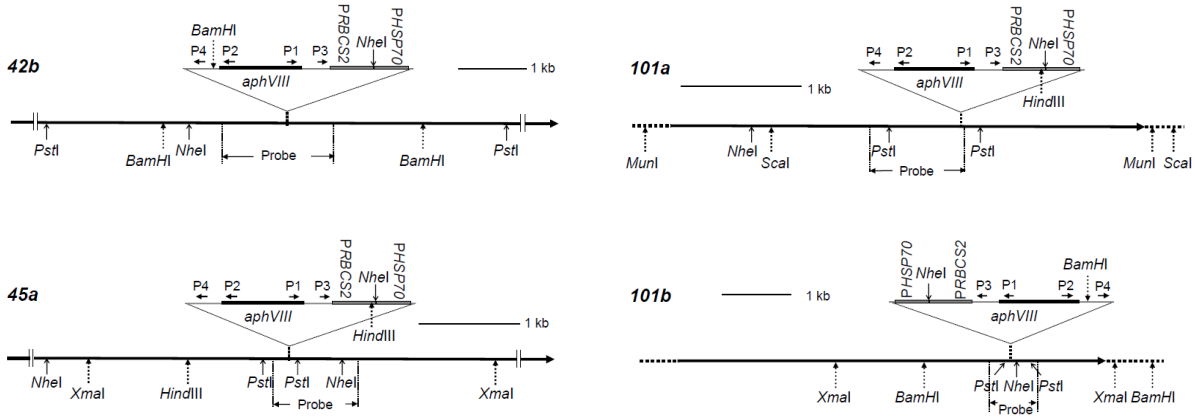
[a] according to the University of California Los Angeles (UCLA) browser for *Chlamydomonas* 454 EST reads (http://genomes.mcdb.ucla.edu/Cre454/);[b] (Stanke and Waack, 2003); [c] obtained from pfam 26.0 (Punta et al., 2012);[d] deduced by ProtParam Tool (Wilkins et al., 1999).

Southern blot analyses on the disrupted genes for all the four mutants were carried out to confirm the insertion site. As shown in Figure 3.2.4, all mutant genomic DNAs revealed altered hybridization patterns as compared to the wild-type, at this confirming an insertion in the identified genes.

Furthermore, the mutation found in *45a* was determined to be linked with the PSII phenotype by BAC clone complementation, as described in section 2.2.8 (data not shown). Additionally, the insertion in *101b* was approved by both BAC clone complementation and co-segregation analysis as described below in chapter 3.4.1.2. The complementation of *42b* by BAC DNA was not successful, either due to the technical reasons, or the identified mutation was not linked to the PSII phenotype. For the *101a* mutant, the co-segregation analysis showed that the mutation found by inverse PCR did not correlate with the PSII phenotype (data not shown), indicating a second mutation in the genome of this mutant. Hence, another molecular method was employed to identify the actual mutation site responsible for the phenotype, which is described in section 3.3.2.

Among the four PSII mutants with identified mutated genes, *42b* and *45a* were not further investigated due to the extremely high molecular weight of the proteins encoded by the mutated genes (306/429 kDa) and their little conservation within the plant kingdom. Therefore, the further molecular and phenotypical analysis was focused on mutants *101a* and *101b* (chapter 3.3 and 3.4).

A



B

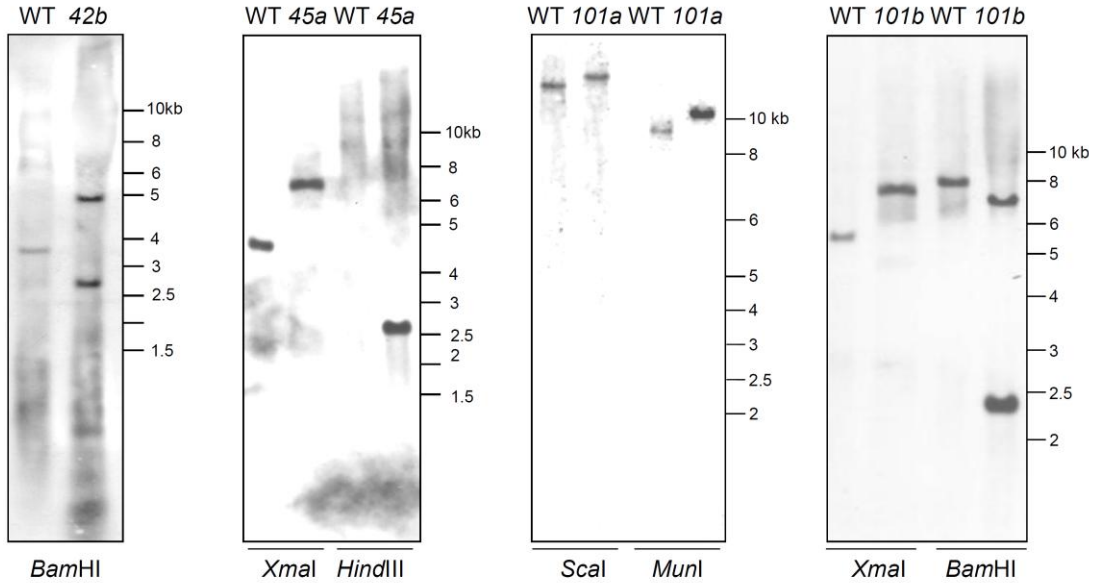


Figure 3.2.4 Southern blot on genomic DNA of *42b, 45a, 101b, and 101a* to confirm the insertion site.
(A) Schematic of the insertion sites of the pBC1 vector in *42b, 45a, 101a,* and *101b*. For simplification, the exon/intron structure is not specified in the mutant genomic DNA, truncated presentation of long genes are indicated by horizontal double lines. The restriction enzymes *Pst*I and *Nhe*I used for inverse PCR are indicated in vertical arrows with real lines, and *BamH*I, *Xma*I, *Hind*III used for Southern blot are indicated in vertical arrows with dashed lines. The primers P1, P2, P3 and P4 used for inverse PCR are indicated by horizontal arrows, corresponding to inv-APHV-fw, inv-APHV-rev, inv-APHV-fw2 and inv-APHV-rev2 respectively, listed in Table 2.6.
(B) Southern blot analyses. 10 µg of genomic DNA was fractionated along with a molecular size marker by 0.8% agarose gels after digestion by appropriate restriction enzymes labeled under each blot. The fractionated DNAs were blotted onto nylon membranes, and hybridized with probes specifically detecting the mutated genes shown in (A). The primers used are listed in Table 2.7.

3.3 Characterization of the *Chlamydomonas reinhardtii 101a* (*ohp2*) mutant

3.3.1 Localization of the mutated gene in the *101a* (*ohp2*) mutant

Since the inverse PCR could not identify the mutation causing the PSII phenotype, another molecular technique based on the principle of map-based cloning was performed to determine the second mutated gene. The *101a* mutant was crossed with the wild-type stain (WT *S24 mt+*), and the progenies from complete and incomplete tetrads were collected for further analyses. The separation of wild-type and PSII deficient progenies were precisely 2:2, which indicated only one mutation in the mutant genome caused PSII phenotype. Interestingly, all of the 47 PSII deficient progenies were found to be mating type minus (*mt-*). This was confirmed by PCRs on mating type specific genes *Mid* (mt-) and *FusI* (mt+), which can be considered as linked gene makers to localize the mutated site. The PCR results shown in Figure 3.3.1 revealed a linkage between the *Mid* gene and the PSII phenotype in all 47 progenies. Therefore, the mutated site in *101a* was suggested to be inside or close to the mating type minus region, which is on the 6 chromosome of *C. reinhardtii*.

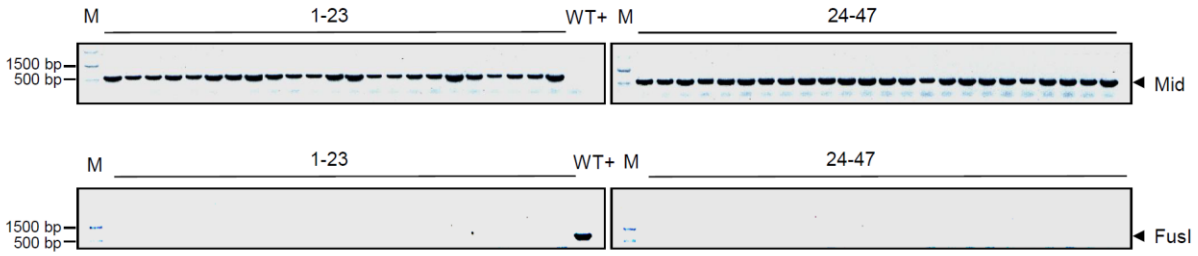


Figure 3.3.1 Detection of *Mid* and *FusI* genes by PCR on progenies with PSII phenotype (1-47) and the mt+ wild-type stain (WT+). Primers were applied for PCR reactions, specific for *Mid* and *FusI* genes (mid-fw: ATGGCCTGTTTCTTAGC, mid-rev: CTACATGTGTTTCTTGACG, fus1-fw: ATGCCTATCTTTCTCATTCT, fus1-rev: GCAAAATACACGTCTGGAAG). Molecular size markers are indicated on the left (M).

As the mutated gene is linked to the mating type minus region, a manual search of this region for potentially mutated candidate genes was performed. Several genes predicted to be related to photosynthesis, were selected for PCR analyses such as *CGL70*, *CRB1*, and *OHP2*. Among these candidates, the *OHP2* gene (gene structure shown in Figure 3.3.2 A), which is approximately 30 kb upstream of the mating type minus region, appeared to be undetectable in all the PSII deficient progenies (Figure 3.3.2 B), whereas the other genes tested were unaltered (data not shown). This *OHP2* gene in *C. reinhardtii* encodes the light induced one

helix protein 2, which will be described in more detail in section 3.3.3. Southern blot analysis with an *OHP2*-specific probe (Figure 3.3.2 A) confirmed the mutation of the *OHP2* gene in the *101a* mutant (Figure 3.3.2 C). Accordingly, the *101a* mutant was renamed as *ohp2* mutant.

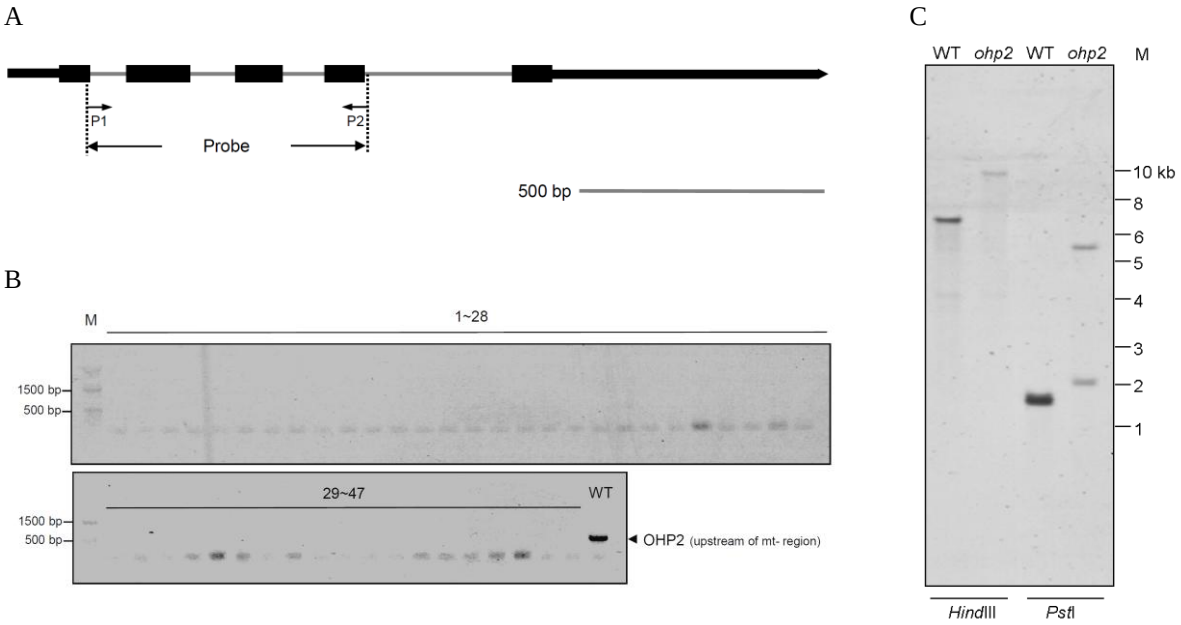


Figure 3.3.2 Molecular analyses of the *ohp2* mutant
(A) Gene model of *OHP2* in *C. reinhardtii.* Exons are shown by black solid boxes, introns by conjunctive black lines, and untranslated regions (UTR) by black solid boxes with smaller size. P1 and P2 (correspond to OHP1-fw2 and OHP1-rev2 listed in Table 2.7) are gene specific primers used to generate the probe for Southern blot shown in (C).
(B) PCR on progenies with PSII deficient phenotypes (numbered 1-47) and the wild-type stain (WT), with primers P1 and P2 specific for the *OHP2* gene, as shown in (A).
(C) Southern blot analysis of genomic DNA from the wild-type and the *ohp2* mutant. 10 µg of genomic DNA were fractionated in a 0.8% agarose gel after digestion by restriction enzyme *Hind*III or *Pst*I, respectively, blotted onto a nylon membrane, and hybridized with the probe indicated in (A)

3.3.2 Complementation of the *ohp2* mutant by *OHP2* cDNA

To further confirm that the loss of functional OHP2 is responsible for the PSII phenotype of the *101a* mutant, complementation studies were performed. The full length cDNA of *OHP2* was PCR amplified and ligated downstream of the strong *PsaD* promoter in the pBC1 vector. The pBC1-*OHP2* construct was transformed into the *ohp2* mutant via electroporation (section 2.2.8). Positive transformants were selected by paromomycin resistance, followed by photoautotrophic growth tests on minimal medium plates. The growth phenotype of positive transformants was restored to that of the wild-type, which was also reflected by reduced chlorophyll fluorescence as stated from almost wild-type-like Q_Y-max values (Figure 3.3.3).

Finally, it could be concluded that, the *OHP2* gene is disrupted in the *ohp2* mutant and responsible for the PSII phenotype.

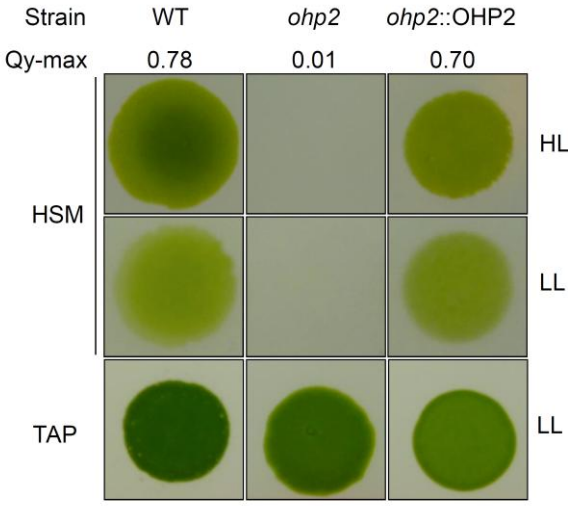


Figure 3.3.3 Photoautotrophic growth test of an *ohp2* complemented strain. The wild-type, *ohp2* and *ohp2* complemented ohp2::OHP2 cells were resuspended in ddH_2O at a concentration of 10^5 cells/mL and spotted onto Petri dishes of TAP or HSM media and grown for 7d under higher light (HL) at 100 µE/m^2/s) or low light (LL) at 30 µE/m^2/s).

3.3.3 Description of OHP2 protein in *Chlamydomonas reinhardtii*

OHP2 encodes a protein of 144 amino acids (Figure 3.3.4) belonging to the family of LIL proteins introduced in section 1.2.1.3.1. The N-terminal 49 amino acids are predicted to represent a chloroplast targeting peptide (cTP) by ChloroP (Emanuelsson et al., 1999). The mature protein without cTP is calculated to have a molecular weight of 11.4 kDa by ExPASy (Gasteiger et al., 2003). The OHP2 protein is predicted to contain a transmembrane helix (amino acids 103-125) by the TMpred server (http://www.ch.embnet.org/software/TMPRED_form.html), which is surrounded by a

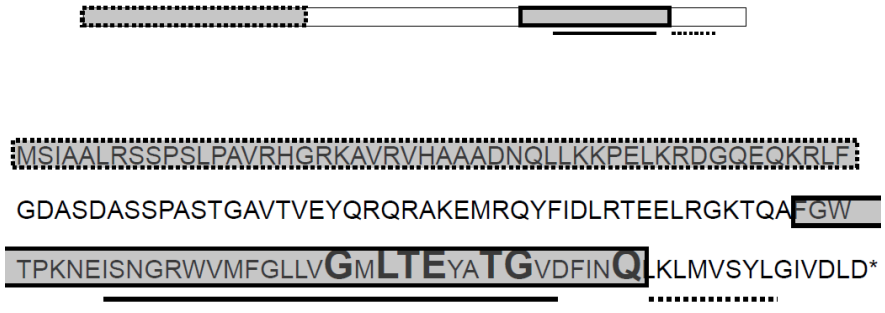


Figure 3.3.4 Protein primary structure of OHP2 in *C. reinhardtii*. Schematic (upper panel) and amino acid sequence (lower panel) of the OHP2 protein are shown. Protein domains were predicted by ChloroP and TMpred. The chloroplast transit peptide is shown as a box with oblique line; the chlorophyll binding region is shown as a solid black box; the predicted transmembrane domain is indicated by a black line under the amino acid sequence, and the dashed line represents the other predicted hydrophobic region. The highly conserved amino acids among the OHP2 homologues are highlighted with bigger size.

predicted chlorophyll binding region, with certain highly conserved amino acid (Adamska et al., 2001), shown in Figure 3.3.4. A second predicted hydrophobic segment presents at the C-terminus between amino acids 131-138, which is suggested to be a putative membrane anchor (Andersson et al., 2003).

By performing BLAST analysis against the non-redundant protein database in NCBI, OHP2 homologues were identified in higher plants, and also in mosses but not in non-photosynthetic organisms. Protein sequences of OHP2 homologues were aligned with ClustalW, and manually modified with BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html). It can be observed that OHP2 and its homologues share low conservation at the N-terminus containing the chloroplast transit peptide (cTP) but a highly conserved putative transmembrane (TM) domain region at the C-terminus (Figure 3.3.5). This TM region overlaps a putative chlorophyll binding domain also described for other LIL proteins (Adamska et al., 2001), which possess several highly conserved residues as indicated in Figure 3.3.4 (lower panel).

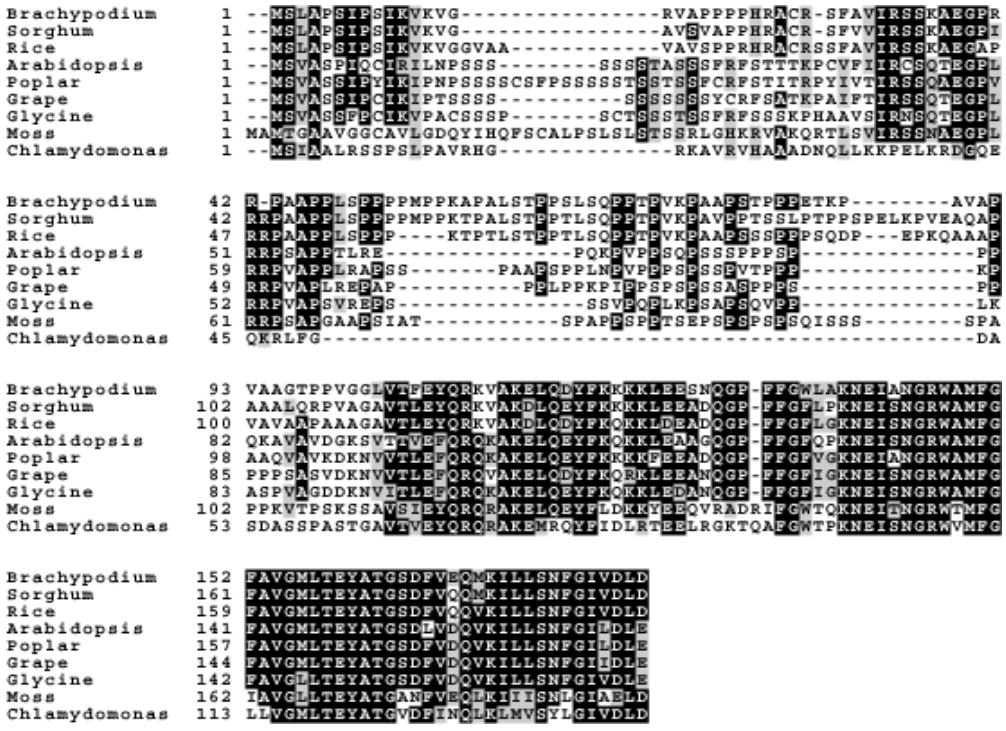


Figure 3.3.5 Sequence alignment of OHP2 homologues in different species. By Blast search, OHP2 homologues in Chlamydomonas (*Chlamydomonas reinhardtii*, GI: 159477110), Arabidopsis (*Arabidopsis thaliana*, GI: 42562501), Moss (*Physcomitrella patens*, GI: 168007372), Grape (*Vitis vinifera*, GI: 359493153), Poplar (*Populus trichocarpa*, GI: 224062862), Rice (*Oryza sativa Japonica*, GI: 297597102), Brachypodium (*Brachypodium distachyon*, GI: 357135380), Glycine (*Glycine max*, GI: 356576079), and Sorghum (*Sorghum bicolor*, GI: 42053441) were obtained from NCBI (www.ncbi.nlm.nih.gov). The multiple sequence alignment was performed by using ClustalW (Thompson et al., 2002) and Boxshade (http://www.ch.embnet.org/software/BOX_form.html). Black boxes indicate strictly conserved amino acids, and gray boxes closely related ones.

3.3.4 Phenotype description of the *ohp2* mutant

To summarize the phenotype analyses described in chapters 3.1 and 3.3 in detail: The Q_Y-max value of the *ohp2* mutant was observed to be 0.01, while that of the wild-type is 0.78, which means the mutant almost completely lost its PSII activity (compare Table 3.2.1). Accordingly, the *ohp2* mutant cannot grow photoautotrophically on HSM media (Figure 3.3.2). Furthermore, there is no detectable accumulation of D2 protein in *ohp2* mutant, whereas RbcL and the PSI core subunit PsaA accumulate to wild-type levels (Figure 3.1.1). In parallel, Northern blot analyses on *psbA* and *psbD* mRNA were carried out. The accumulation of *psbA* and *psbD* transcripts did not show any differences between the wild-type and the *ohp2* mutant (Figure 3.1.2), which demonstrates that the OHP2 protein is not involved in transcriptional regulation or transcript stabilization, but in later steps of PSII subunit synthesis or assembly.
In order to verify the reason which can explain the loss of PSII in the *ohp2* mutant, *in vivo* labeling experiments were performed on wild-type, *ohp2* mutant, and other PSII deficient mutants as controls. As shown in Figure 3.1.3 B, the *ohp2* mutant has almost no detectable D1 protein as compared to the wild-type, but normal amounts of other subunits of PSII and ATPase, which indicates that specifically the normal transcribed *psbA* transcript could not be translated into D1 protein in the *ohp2* mutant, or the newly synthesized D1 protein is not stable. The pattern of thylakoid protein synthesis of the *ohp2* mutant is similar to that of the *Fud7* mutant, which is a *psbA* deletion mutant. Taken together, the results suggest that OHP2 might be involved in translation or stabilization of D1 subunits.

3.4 Characterization of the *Chlamydomonas reinhardtii 101b* (*clr24*) mutant

The *101b* mutant was renamed as *clr24* since the *CLR24* gene was shown to be disrupted in the *101b* mutant (chapter 3.2). In the following, co-segregation and complementation assays were performed to confirm that the mutated *CLR24* gene actually causes the PSII phenotype in the *101b* mutant. Furthermore, different biochemical experiments were carried out to investigate the precise phenotype of *clr24*. As CLR24 belongs to the OPR protein family, whose members are described to be involved in organellar RNA metabolism, this chapter also includes the analysis of putative RNA targets of the CLR24 protein to elucidate its function.

3.4.1 The mutation in *CLR24* causes the PSII phenotype

To confirm a correlation of the PSII phenotype and the mutation in the *CLR24* gene co-segregation and complementation assays were performed on the *101b* mutant.

The co-segregation analysis was carried out in collaboration with Dr. Olivier Vallon (CNRS/Universit é Pierre et Marie Curie, Institut de Biologie Physico-Chimique, Paris). The *clr24* mutant which is an *mt-* strain was crossed with *mt+* wild-type strain. Tetrads were separated in the 4-progeny-stage, and 37 progenies from 10 complete and incomplete tetrads were collected for further analysis. Wild-type progenies and PSII deficient progenies showed a 2:2 segregation, which confirmed that only one single gene involved in photosynthesis is interrupted in the *clr24* mutant. In addition, PCR analyses and Q_Y-max measurements for all progenies confirmed a correlation of the PSII phenotype and the mutation in *CLR24* (Figure 3.4.1).

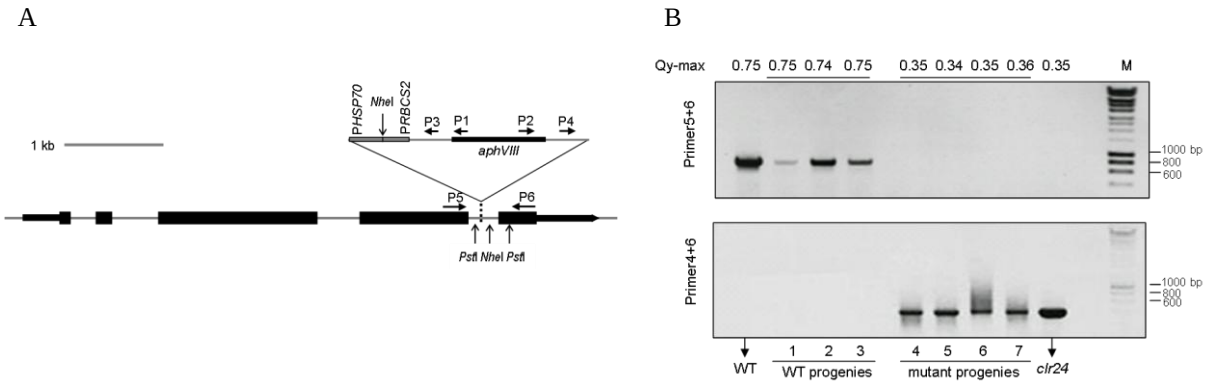


Figure 3.4.1 Co-segregation analysis of *clr24* mutant.
(A) Insertion site of the mutagen in the *CLR24* gene in the *clr24* mutant. Exons are shown as black boxes and introns as gray lines. The locations of both the *aphVIII* gene and the promoters of the mutagen are indicated, together with the primers and restriction enzyme restriction sites used for inverse PCR (section 2.2.4.5).
(B) PCR analysis of 37 progenies from *clr24* x WT crossing. 10 tetrads were analyzed by PCR with primers shown in (A) and listed in Table 2.6. Here, exemplarily shown only 7 out of 37 progenies (1-7).

To further verify the role of CLR24 in photosynthesis, complementation studies were performed (Figure 3.2.4). For this purpose, BAC DNA containing the genomic *CLR24* region was transformed into the *clr24* mutant (section 2.2.8). The successful integration of the *CLR24* gene was confirmed by PCR (Figure 3.4.2 A). The complemented strains *clr24*::CLR24 showed higher Q_Y-max values (0.65~0.72) as compared to the *clr24* mutant (~0.35), with a restored ability to grow photoautotrophically on minimal medium (Figure 3.4.2). Taken together, complementation and co-segregation results clearly confirmed the mutation in the *CLR24* gene to be responsible for the photosynthesis defect of the *101b* mutant.

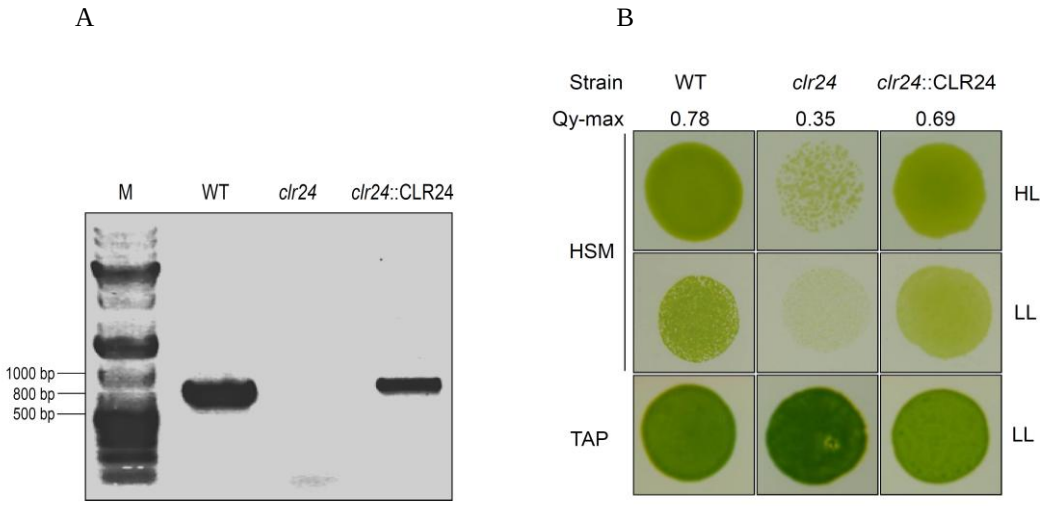


Figure 3.4.2 Complementation of the *clr24* mutant.
(A) Confirmation of *CLR24* integration into the genome of the *clr24* mutant after BAC clone transformation. Positive *clr24* complemented line (*clr24*::CLR24), wild-type (WT), and the *clr24* mutant (*clr24*) were screened by PCR with primers P5 and P6 shown in Figure 3.4.1 A
(B) Photoautotrophic growth test of a complemented strain on HSM plates. Cells of wild-type, *clr24* mutant and complement strain were resuspended in ddH_2O at a concentration of 10^5 cells/mL and spotted onto Petri dishes of TAP and HSM media, grown for 7d under low light (LL, 30 µE/m^2/s) or high light (HL, 100 µE/m^2/s) conditions, respectively. Q_Y-max values are indicated.

3.4.2 Description of the CLR24 protein in *Chlamydomonas reinhardtii*

The *CLR24* gene encodes a protein of 1330 amino acids (138 kDa) and belongs to the OPR protein family (section 1.3.2.3). The N-terminal 74 amino acids are predicted by ChloroP as plastid targeting sequence (Figure 3.4.3 A) and the mature protein is calculated to have a molecular weight of 132 kDa. CLR24 contains 14 OPR motifs, of which each is predicted to form two α-helices (Figure 3.4.3 A). A multiple alignment of these 14 OPR repeats reveals, for all of the repeats a higher similarity to the consensus sequence in the first predicted helix

containing the LWALA motif than in the second one (Figure 3.4.3 B, compare section 1.3.2.3).

A

B

```
Repeat1   : SALPQELSNLALGLAKLGYREVPLWAAIIAAGKARLP--
Repeat2   : AFKPQELHNLAWAVAAASQDRSMISAAVQAALPQLG---
Repeat3   : AFTPSGLSNLLWACATAQCHVEELFDGAAAALLR-----
Repeat4   : QLNSQDVANTAWAFAKVQHPHPGLMRHLGGLVLRAATG-
Repeat5   : GVATQELVNVLWAFASMPPQGLGLPDAAAGGVPAQGRG-
Repeat6   : DLTPQGASNALWAAGRLQPCPVPPDALADALRAASTRAA
Repeat7   : SMSDQELANALWAAGELRGAGHYVPPAAVAPLFAAACCP
Repeat8   : ATPAAGVAQLVSAAVKLRLVGSQHMDALAKRVMRGLG--
Repeat9   : SLGPQELCVLAAAVAEAVHVAAYCNPILLNGLANAAVA-
Repeat10  : RLDPQGLSTLLWAFARAGKHYHGPLTTTICRVAAPRL--
Repeat11  : EFSDLELSNLVWALAVLKCQDRQLLVRAARVLVGRVRLR
Repeat12  : LLTPHEVVQVLWSVAYHSHSCPELLDAAAPAIASRLGR-
Repeat13  : RFCPWDASVVAWAYAKLDHPHRDLFESLQHHALRYGS--
Repeat14  : RYKEPCLLRLVWACAQLQLHVREPLLAQLHALRTGRSR-
Consensus : GFKPQELSNLLWALAKLGHQPPPALLDALAAAAAARLP-
```

Figure 3.4.3 Features of the CLR24 protein of *C. reinhardtii*
(A) Schematic (upper panel) structure and amino acid sequence (lower panel) of CLR24. The plastid transit sequence predicted by ChloroP is shown as a dashed box; the OPR repeats obtained by alignment with the OPR protein consensus sequence are shown as solid boxes. The predicted helical structures obtained by Jpred (www.compbio.dundee.ac.uk/www.jpred) are indicated as black lines below the amino acid sequence.
(B) Multiple sequence alignment between the OPR consensus sequence and CLR24 OPR repeats was performed by Genedoc. Dark gray boxes indicate more conserved amino acids, and light gray boxes less conserved ones.

3.4.3 The formation of PSII dimers and supercomplexes is affected in the *clr24* mutant

As already revealed in the initial analysis of all investigated PSII mutants in chapter 3.2, the *clr24* (*101b*) mutant showed a reduced D2 protein accumulation, only weak photoautotrophic growth, and increased chlorophyll fluorescence as indicated by a Q_Y-max value of 0.35 (compare Table 3.2.1). However, no defects were observed in transcript accumulations or in thylakoid protein synthesis. These results indicate that the translation apparatus in the *clr24* mutant is functional as in the wild-type, and CLR24 could be involved in the post-translational regulation of PSII subunits such as assembly or stabilization of PSII complexes. Therefore, further phenotypic analyses were carried out to gain insights into the precise function of the CLR24 protein.

As introduced before, PSII complexes are multi-protein complexes, which are composed of more than 20 subunits, including the core subunit D1 and D2 (section 1.2.1.1). All the subunits are stabilized upon successful assembly into a complex. In case of assembly defects, their accumulation is affected due to the degradation mechanisms (Choquet et al., 2001). In order to verify the reason for the reduced accumulation of the D2 protein in the *clr24* mutant, photosynthetic complexes of the wild-type and the *clr24* mutant were analyzed by blue native PAGE (section 2.2.5.8, Figure 3.4.4). The results obtained indicate that the inter-complex stoichiometry is largely changed in the *clr24* mutant, and all forms of PSII complexes are reduced dramatically, such as PSII monomers, dimers, and supercomplexes (Figure 3.4.4 A). In the second dimension, denaturing SDS-PAGE was carried out to analyze the abundance of individual subunits in different complexes. In the *clr24* mutant, PSII subunits were severely reduced in the PSII monomer, and only traces of PSII dimers can be observed, while the super molecular structure of PSII was not detectable (Figure 3.4.4 B).

In order to confirm the results obtained from stained gels in Figure 3.4.4 B, immunoblot analyses were carried out on the second dimension, by using antibodies against D2 and CP43. As shown in Figure 3.4.4 C, the PSII dimers are detected at much reduced levels and supercomplexes are completely undetectable. Furthermore, the PSII monomers also show a reduced accumulation in the *clr24* mutant. The antenna protein CP43, which is involved in PSII assembly at a later stage, is also reduced in PSII monomers (Figure 3.4.4 C). Although in the one-dimensional SDS-PAGE gel of *in vivo* pulse labeled proteins, there was no change of protein synthesis detectable (Figure 3.1.3 B), a second dimension SDS-PAGE gel showed obvious differences (Figure 3.4.5). The ^{35}S labeled thylakoid proteins were obtained from

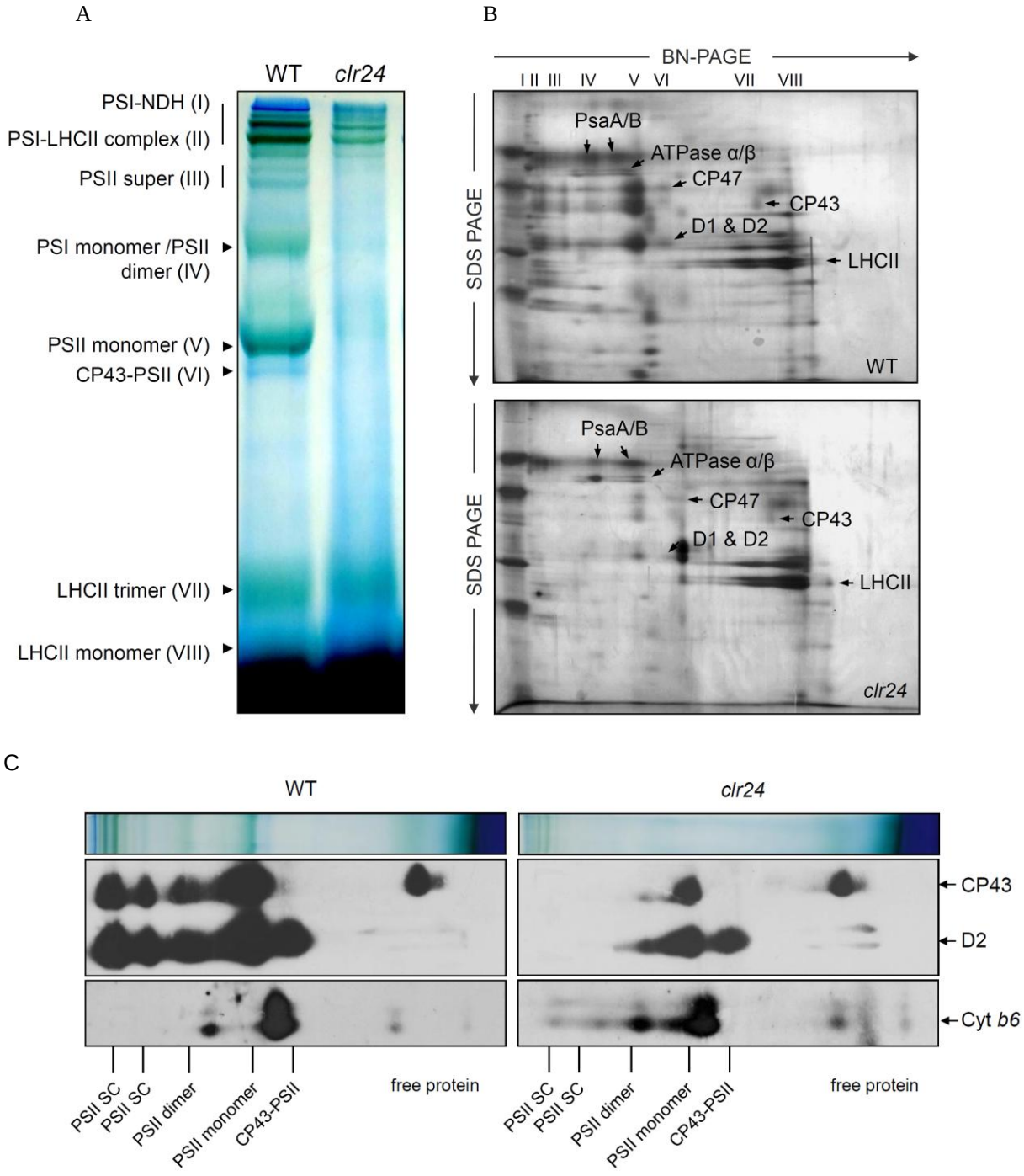


Figure 3.4.4 Accumulation of PSII complexes

(A) BN-PAGE analyses of thylakoid photosynthetic complexes. Thylakoids from wild-type and the *clr24* mutant were solubilized with 1.5% (w/v) β-DM. Extracts were fractionated by 4.5%-15% BN-PAGE. Complexes detected were identified in accordance with previously published profiles (Granvogl et al., 2006; Peng et al., 2008; Schwenkert et al., 2006): PSI-NDH supercomplex (PSI-NDH; I), PSI-LHCII complex (II), PSII supercomplexes (PSII super; III), PSI monomers and PSII dimers (PSI monomer and PSII dimer; IV), PSII monomers (PSIImonomer; V), CP43-free PSII monomers (CP43-PSII; VI), trimeric LHCII (LHCII trimer; VII), and monomeric LHCII (LHCII monomer; VIII).

(B) 2D SDS-PAGE separation of thylakoid protein complexes. Individual lanes from BN-PAGE gels as in (A) were fractionated in the presence of 30% urea by gel electrophoresis on 15% polyacrylamide gels. Gels were visualized by silver staining. The identity of relevant proteins is indicated by arrows.

(C) Detection of PSII assembly complexes by immunoblot analyses of 2D BN/SDS gels as in (B) with antibodies against D2 and CP43. Positions of PSII complexes PSII SC, PSII supercomplexes; PSII dimers; PSII monomers and CP43-free PSII monomers are indicated. Cyt *b6* antibody was applied as the loading control.

both the wild-type and the *clr24* mutant as described (section 2.2.5.9). The labeled thylakoids were separated on blue native PAGE, followed by 2D SDS-PAGE analysis. These results show that D1/D2 proteins are synthesized at normal levels, but cannot be efficiently assembled into higher order complexes. Accordingly, there is almost no detectable D2 in PSII dimers and supercomplexes. From these results, it can be concluded that the formation of PSII dimers is affected in the *clr24* mutant. Taken together with previous studies on PSII super molecular organization which report that most functional PSII complexes are in the dimer form, it can be assumed that the defect on PSII dimer formation in the *clr24* mutant causes the PSII deficient phenotype.

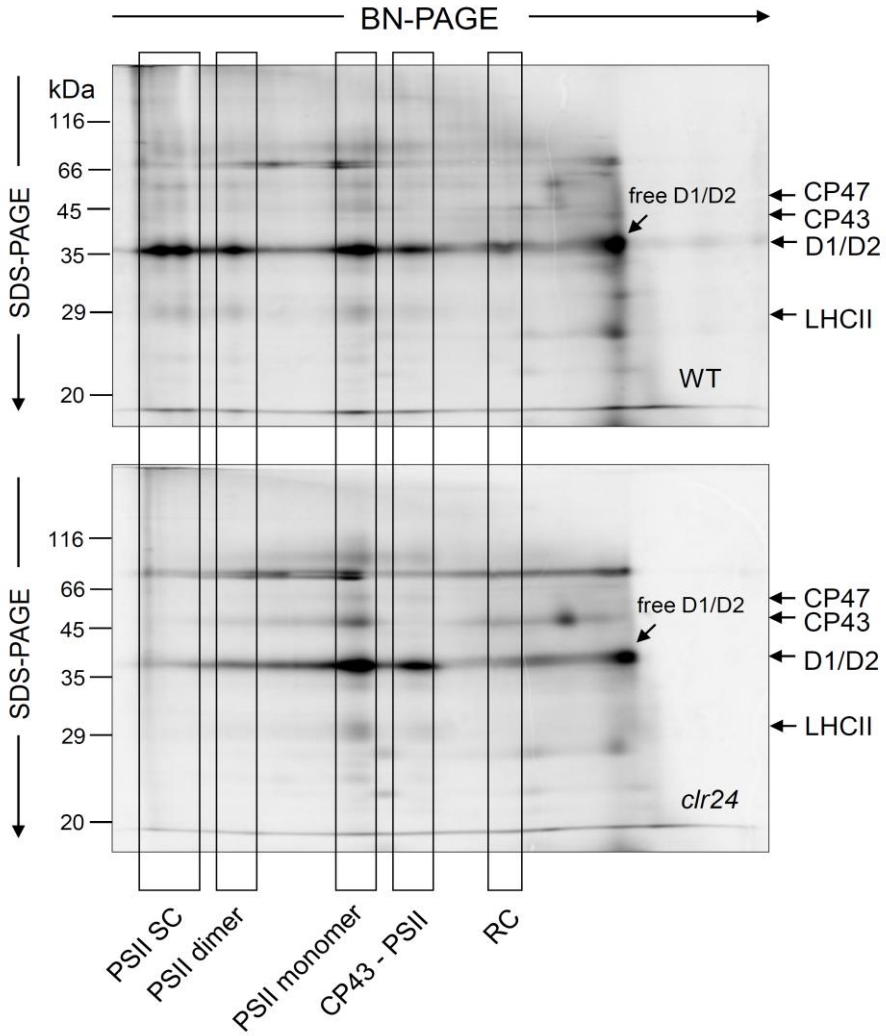


Figure 3.4.5 PSII complexes assembly detected by *In vivo* labeling. Thylakoid membrane proteins of wild-type and *clr24* strains were labeled with ^{35}S as described in Figure 3.1.3 B, and fractionated by 2D BN/SDS-PAGE. The labeled complexes were visualized by autoradiography. The positions of different PSII assembly complexes are indicated on the bottom of the figure.

3.4.4 Functional analyses of the CLR24 protein

According to the results obtained (section 3.4.3), the CLR24 protein functions in PSII dimer formation. However, the precise mechanism by which CLR24 influences this PSII assembly step remained unclear. Based on the assumption that CLR24 is involved in chloroplast RNA metabolism, this chapter focuses on the identification of a CLR24 specific RNA target.

3.4.4.1 *PsbI* transcripts are not detectable in the *clr24* mutant

As transcripts of PSII subunits, like *psbA* or *psbD*, were normally synthesized and translated in the *clr24* mutant, the function of CLR24 in PSII assembly might be rather indirect. According to previous studies, PSII dimer formation in *C. reinhardtii* requires the involvement of several subunits with low molecular weight, which are encoded by the chloroplast genome (Rochaix, 2011, section 1.2.1.3). Since OPR proteins are predicted to have an RNA-binding domain, it was of particular interest to investigate the involvement of CLR24 in PSII dimer formation via interaction with transcripts of those small subunits.
In order to prove the above mentioned hypothesis, six transcripts of small chloroplast encoded subunits, which have been characterized to participate in PSII dimer formation, including *psbI*, *psbK*, *psbL*, *psbM*, *psbT*, and *psbZ*, were investigated via Northern analyses in the *clr24* mutant along with wild-type. No difference in the accumulation of *psbK*, *psbL*, *psbM*, *psbT*, and *psbZ* was detected in the *clr24* mutant as compared to wild-type. But interestingly, it was observed that *psbI* transcripts are undetectable in the *clr24* mutant (Figure 3.4.6). The *psbI* mRNA encodes a small subunit with 37 amino acids, which is described to be involved in PSII dimer formation in tobacco (Schwenkert et al., 2006).

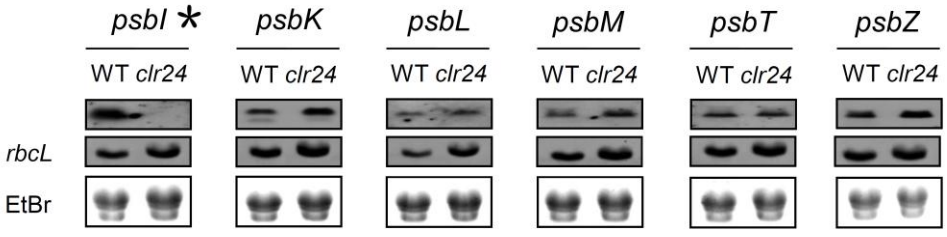


Figure 3.4.6 Transcript accumulation analyses on chloroplast genome encoded small subunits involved in PSII assembly. 5 µg of total RNA from wild-type (WT) and the *clr24* mutant (*clr24*) were hybridized with probes specific for *psbI, psbK, psbL, psbM, psbT, psbZ,* respectively. *rbcL* transcripts and the ethidium bromide stained agarose gel (EtBr) were used as a loading control. All the signals detected are mature monocistronic transcripts. The change in *psbI* transcript accumulation is indicated by a star.

3.4.4.2 Altered processing of *atpA-psbI-cemA-atpH* polycistronic transcripts in the *clr24* mutant

The *psbI* mRNA is co-transcribed with *atpA*, *cemA*, and *atpH*, which encode alpha-subunit of the coupling-factor-1 (CF1) ATP synthase, a chloroplast envelope membrane protein and subunit III of the CF0 ATP synthase respectively. The *psbI* mRNA can occur as monocistronic, dicistronic, as well as polycistronic transcripts, due to three promoters used during transcription (Drapier et al., 1998, compare Figure 3.4.7 A). Since the mature *psbI* monocistronic transcript is undetectable in *clr24* mutant (Figure 3.4.6), Northern analyses of

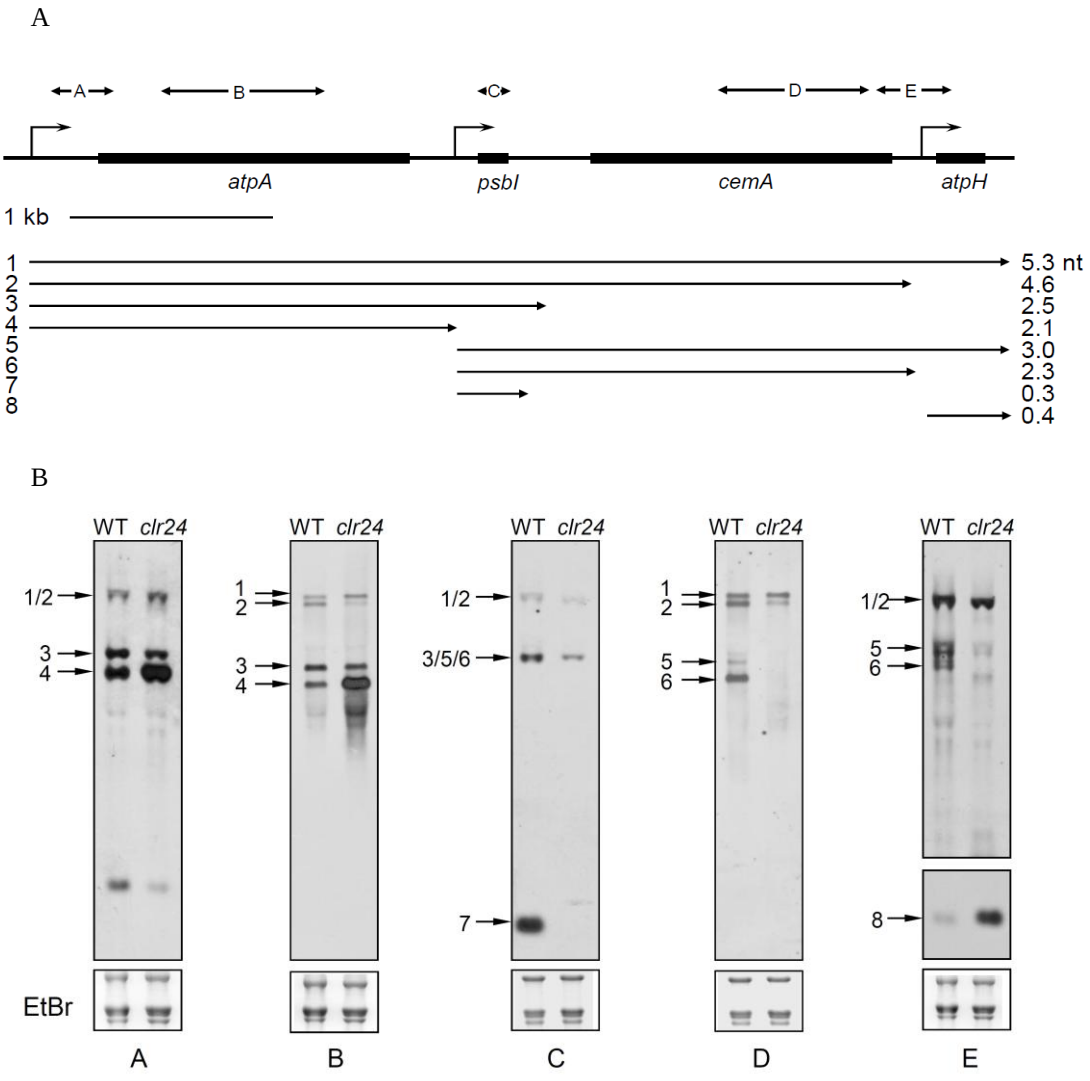


Figure 3.4.7 Transcript abundance of the chloroplast *psbI* gene cluster.
(A) Map of the *psbI* gene cluster. Genes are indicated by solid black boxes and mapped promoters by bent arrows (Drapier et al., 1998). Transcripts are depicted as black horizontal arrows and labeled by numbers 1-8 according to Drapier at al. (1998) . Corresponding transcript sizes are indicated at the right. Positions of probes used in (B) are shown above the map (A-E).
(B) Northern blot analysis of the *psbI* gene cluster. 5 µg of total RNA from wild-type and the *clr24* mutant were hybridized with 5 different probes (A-E). The lower autoradiogram in E is a weaker exposure from the same blot as upper part to get clearer resolution. The EtBr-stained gels of rRNA (EtBr) are used as a loading control.

the entire *atpA-psbI-cemA-atpH* gene cluster were carried out to investigate its processing. Probes for Northern analyses were generated either at the coding region of *atpA, psbI, and cemA* (B, C, D in Figure 3.4.7 A), or at the processing sites (A, E in Figure 3.4.7 A) to detect different processed transcripts. The abundance of the polycistronic *atpA-psbI-cemA-atpH* transcript in *clr24* seemed to be slightly increased (Figure 3.4.7 B, probes B and D).

In contrast, the *psbI-cemA* dicistronic transcripts (arrow 6 in Figure 3.4.7 A, B, probe D, E) and the *psbI-cemA-atpH* tricistronic transcripts (arrow 5 in Figures 3.4.7 A, B, probe D, E) together with the *psbI* monocistronic transcripts (arrow 7 in Figure 3.4.7 A, B, probe C) were undetectable in the *clr24* mutant. The *atpA-psbI* dicistronic transcript (arrow 3 in Figure 3.4.7 A, B, probe A, B) and the *atpA-psbI-cemA* tricistronic transcript (arrow 2 in Figure 3.4.7 A, B, probe B, D) were slightly reduced as compared to the wild-type. On the contrary, the accumulation of transcripts without *psbI*, such as *atpA* (arrow 4, Figure 3.4.7 B, probe A, B) and *atpH* (transcript 8, Figure 3.4.7 B, probe E) monocistronic transcripts were increased significantly. Taken together, the processing of the entire *atpA-psbI-cemA-atpH* transcript is affected in the *clr24* mutant. The absence of mature *psbI* (monocistronic) and *cemA* (dicistronic) transcripts might lead to the PSII deficient phenotype in the *clr24* mutant.

3.5 Characterization of *Arabidopsis thaliana AtRAP-1* mutant

According to BLAST results, the AtRAP protein (At2g31890) is the only member of the OPR family found in *A. thaliana* what makes the elucidation of its function particularly interesting (compare section 1.3.2.3).

3.5.1 Description of the AtRAP protein in *Arabidopsis thaliana*

AtRAP consists of 671 amino acids, of which the N-terminal 78 aa are predicted by ChloroP to be a plastid targeting sequence (Figure 3.5.1 A). The mature protein is calculated to have a molecular weight of 70 kDa. Four OPR repeats are identified which are, like OPR repeats from *C. reinhardtii*, predicted to form two α - helices (Figure 3.5.1 A, B).

Figure 3.5.1 Features of the *A. thaliana* AtRAP protein.
(A) Schematic (upper panel) and amino acid sequence (lower panel) of the protein structure of AtRAP. The plastid transit sequence predicted by ChloroP is shown as a dashed box. The OPR repeats obtained by alignment with the OPR protein consensus sequence are shown as real line boxes. The RAP domain is shown as a longer dashed box. Helices predicted by Jpred (www.compbio.dundee.ac.uk/www.jpred) are marked with straight lines below the sequence.
(B) Multiple sequence alignment between OPR consensus sequence and four predicted AtRAP OPR repeats was manually defined and illustrated by Genedoc. Black boxes indicate conserved amino acids in all repeats, dark and light gray boxes indicate conserved amino acids in 80 or 60%, respectively, of the aligned residues.

Additionally, a so-called RAP domain, often seen at the C-terminus of OPR proteins (A. Bohne, personal communication) is found at aa positions 608-668 (Figure 3.5.1 A). This RAP domain (RNA-binding domain abundant in Apicomplexans) is reported to be particularly abundant in apicomplexans and might mediate a range of cellular functions through a potential interaction with RNA (Lee and Hong, 2004).

3.5.2 Growth characteristics and photosynthetic performance of the *AtRAP* mutant

Two mutant alleles were identified for *AtRAP* by screening the T-DNA Express database, of which one has a T-DNA insertion at the 5' UTR, the other one inside the third exon (Figure 3.5.2 A). Homozygous mutants were obtained from the T3 generation and controlled by PCR

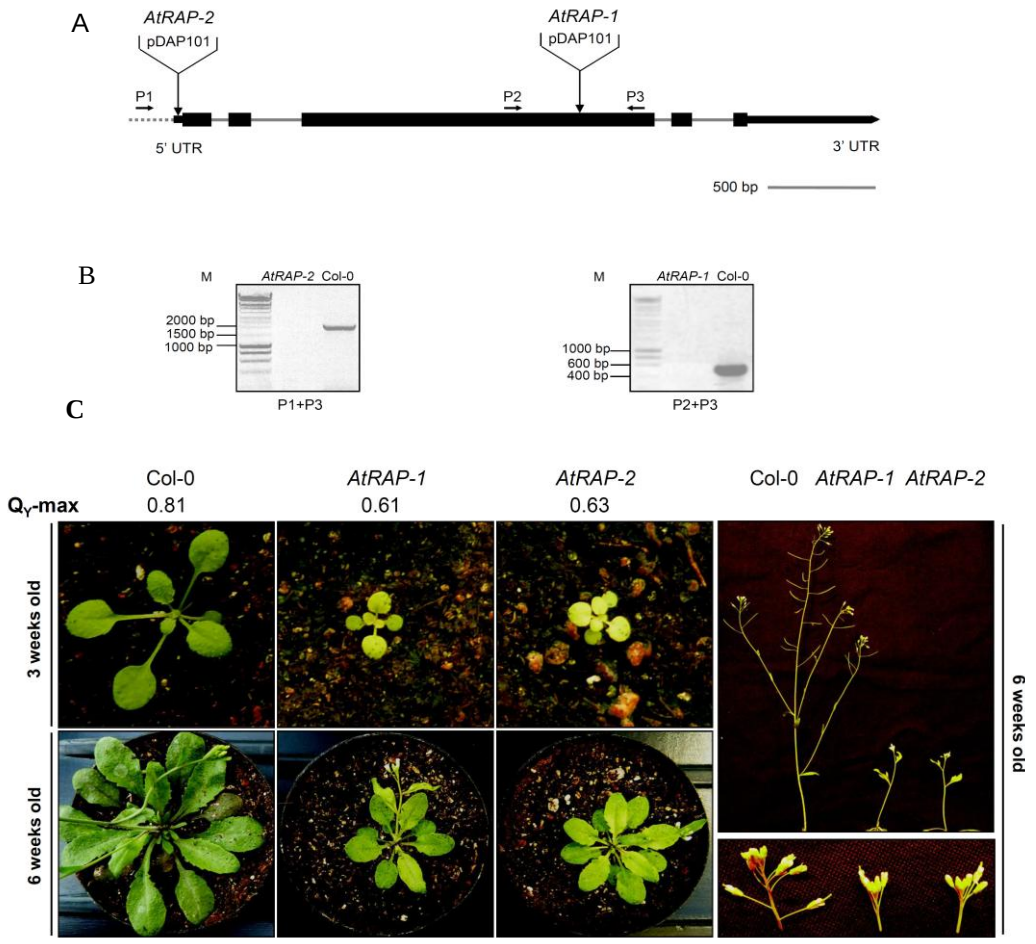


Figure 3.5.2 Location of insertion in *AtRAP* mutant alleles and mutant phenotypes.
(A) Schematic gene structure of *AtRAP*. Exons are shown as black boxes and introns as gray lines. The sites of T-DNA insertions are depicted for both of the mutants (*AtRAP-1*, SAIL_1223_C10; *AtRAP-2*, SAIL_1225_B10). Primes used for detecting homozygous mutants are indicated by arrows.
(B) PCR reactions for detecting homozygous T-DNA insertion mutants. The gene specific primers used are shown in (A), P1, P2 and P3 are corresponding to ara-101b-fw, 31890-fw and 31890-rev respectively, which are listed in section 2.1.7. DNA molecular marker was labeled on the left side.
(C) Growth phenotype of two independent insertion mutant lines. *AtRAP-1*, *AtRAP-2*, and corresponding wild-type (Col-0) grown for three or six weeks, respectively, under long day conditions in the green house**.** Q_Y-max values indicated on top were measured by using 3-week-old plants.

for homozygosity, as shown in Figure 3.5.2 B. Both mutant alleles for *AtRAP* display a reduction in leave size and show pale-green pigmentation, when grown in the greenhouse with long-day illumination (16h light, 8h dark). The photosynthetic activity is affected in both of the mutants, as indicated by lower Q_Y-max values as compared to the wild-type (Figure 3.5.2 C). After 6 weeks growing, mature leaves of *AtRAP* mutants started to turn green like the wild-type, but retained the growth retardation phenotype. Even though siliques from both of T-DNA lines have smaller sizes, seeds could be obtained from homozygous plants (Figure 3.5.2 C).

3.5.3 Phenotypic characterization of *Arabidopsis thaliana AtRAP-1* mutants

The accumulation of core proteins from photosynthetic complexes was investigated in the *AtRAP-1* mutant along with the wild-type. Since both mutant lines, *AtRAP-1* and *AtRAP-2*, possess identical growth phenotypes, further analyses were all applied on *AtRAP-1*. To investigate the accumulation of PSII, PSI, and the RuBisCo complex, representative proteins, including the chloroplast encoded D1, D2, PsaA and RbcL, as well as nucleus-encoded LHCII proteins, were detected by immunoblot analysis or Coomassie staining (Figure 3.5.3). Comparing with wild-type plants, 3-week-old *AtRAP-1* mutants have dramatically reduced amounts of all chloroplast encoded proteins (Figure 3.5.3), which could lead directly to a growth retardation. However, the reduction level of the D1, D2 and PsaA proteins becomes less severe after 6 weeks of growth, which also corresponds to the slightly restored phenotype for the 6-week-old mutants. Interestingly, no alteration of protein accumulation compared to the wild-type was observed in *AtRAP-1* for the nucleus encoded LHCII.

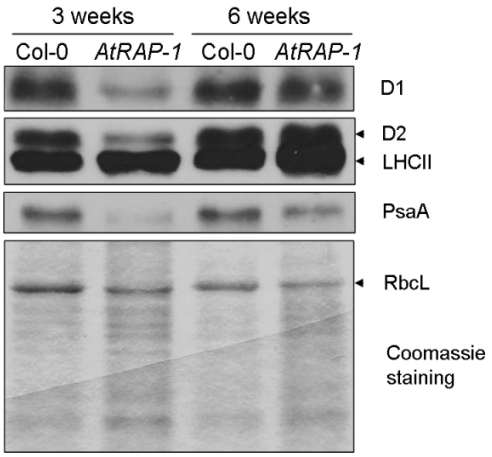


Figure 3.5.3 Accumulation of abundant chloroplast proteins. 30 µg total protein extracts of 3-week-old and 6-week-old plants from wild-type (Col-0) and *AtRAP-1* plants were separated on a 12% SDS-PAGE gel. Antibodies against PSI core subunit PsaA, PSII core subunit D1, D2, and LHCII were applied to detect respective proteins. The RbcL protein was detected by Coomassie staining of the gel.

In order to investigate if defects in the assembly of chloroplast complexes are responsible for the observed reduction of protein accumulation, an analysis of thylakoid proteins from 3-week-old wild-type and *AtRAP-1* plants was performed via blue native PAGE (Figure 3.5.4).

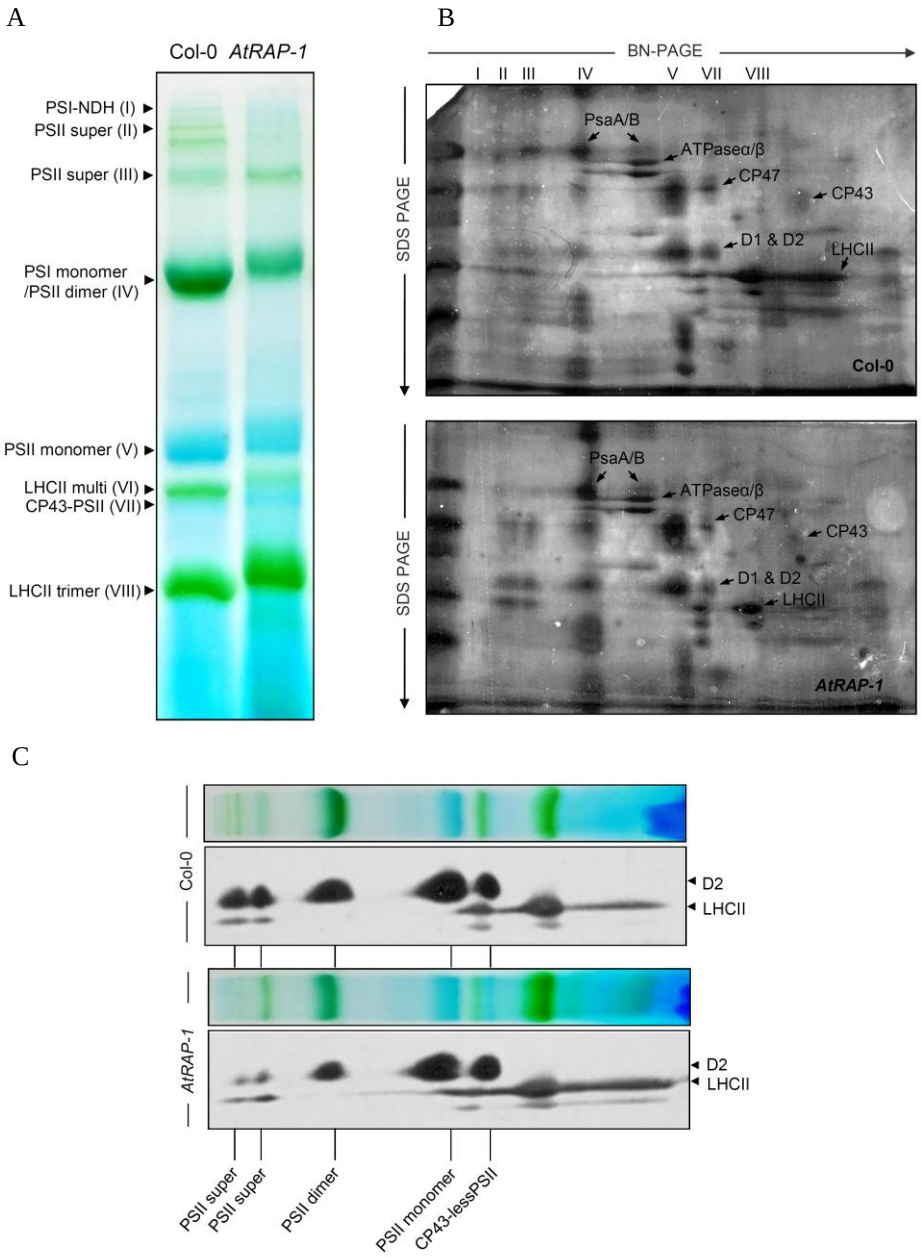


Figure 3.5.4 Analysis of thylakoid membrane complex assembly in the wild-type and the *AtRAP-1* mutant
(A) Thylakoids from wild-type and the *AtRAP-1* mutant were solubilized with 1.5% (w/v) β-DM. The extracts were then fractionated by 4.5%-14% BN-PAGE. Bands detected were identified by comparison with specific protein complexes according to previously published profiles (Granvogl et al., 2006; Schwenkert et al., 2006; Peng et al., 2008): PSI-NDH supercomplex (PSI-NDH; I), PSII supercomplexes (PSII super; II and III), PSI monomers and PSII dimers (PSI monomer and PSII dimer; IV), PSII monomers (PSII monomer; V), multimeric LHCII (LHCII multi; VI), CP43-less PSII monomers (CP43-PSII; VII), trimeric LHCII (LHCII trimer; VIII).
(B) 2D BN/SDS-PAGE separation of thylakoid protein complexes. Individual lanes from BN-PAGE gels as described in (A) were separated in the presence of 30% urea by electrophoresis on 15% polyacrylamide gels, and visualized by silver staining. The identity of relevant proteins is indicated by arrows.
(C) Detection of PSII complexes by immunoblot analysis of 2D BN/SDS gels as in (B) with antibodies against D2 and LHCII. The positions of PSII complexes (PSII supercomplexes; PSII dimers; PSII monomers and CP43-PSII, CP43-less PSII monomers are indicated).

As shown in Figure 3.5.4 A and B, all photosynthetic complexes could be detected in the *AtRAP-1* mutant even though particularly the first dimensional gel (Figure 3.5.4 A) showed a reduction of higher molecular weight complexes like PSI-NDH or PSII supercomplexes. However, the second dimensional gel revealed that all mutant thylakoid membrane complexes were assembled normally, as no obvious differences compared to the wild-type were detected (Figure 3.5.4 B).

This observation was confirmed further by the immunoblot analysis on the second dimension gel (Figure 3.5.4 C). The D2 antibody was applied to detect the PSII complexes, whereas LHCII was used as a loading control. All functional PSII complexes are assembled properly, but are decreased in accumulation, at this confirming the steady state protein levels detected in the one dimensional immunoblot analysis (Figure 3.5.3).

Since the reduced accumulation of chloroplast proteins is not caused by assembly defects of photosynthetic complexes indicated by blue native PAGE (Figure 3.5.4), the translation of chloroplast encoded proteins might be affected. To test this, *in vivo* pulse labeling experiments of thylakoid proteins were carried out on 3-week-old wild-type and *AtRAP-1* plants. As shown in Figure 3.5.5, a significant reduction in the synthesis rate of D1, D2, CP43 and CP47 proteins could be observed, which together compose the PSII monomer (Figure 3.4.5). Interestingly, α/β subunits of ATPase synthesis is not affected, rather has increased in the *AtRAP-1* mutant. It is a possible that translational regulation of ATPase is somehow different from that of other abundant chloroplast encoded proteins, like D1, D2, CP43, CP47 detected also by *in vivo* labeling.

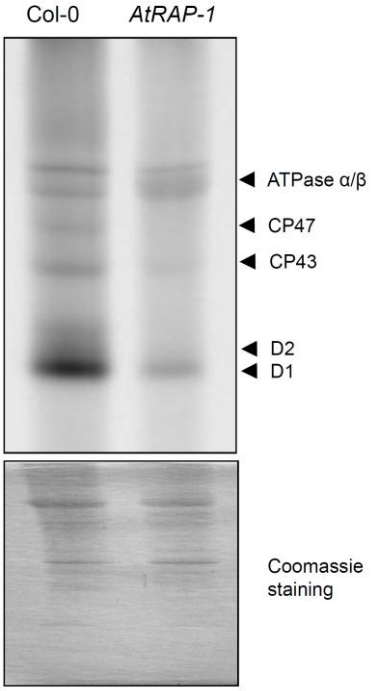


Figure 3.5.5 *In vivo* labeling of 3-week-old wild-type and *AtRAP-1* plants. 3 weeks-old plants were used for *in vivo* labeling studies. Leaves from Col-0 and *AtRAP-1* were labeled with ^{35}S-methionine for 30 min. Cycloheximide was used to inhibit cytosolic translation. Thylakoids extracts from labeled leaves were separated on tricine-SDS-PAGE. Major proteins (D1, D2, CP43, CP47, and ATPase α/β) from photosynthetic complexes are indicated by arrows.

In order to determine the reason for reduced translation rates of chloroplast encoded proteins in the *AtRAP-1* mutant, the accumulation of *psbA* and *rbcL* transcripts, was investigated via RNA gel blot analyses using RNA from both 3-week-old and 6-week-old plants of *AtRAP* mutant and wild-type. As compared to wild-type, no analyzed transcript showed a different accumulation, especially in the 3-week-old *AtRAP-1* mutants, which possesses a severe phenotype (Figure 3.5.6). Taken together all these results, it is obvious that the reduction in the translation rate of chloroplast encoded proteins is due to a chloroplast translation defect in *AtRAP-1* mutants. Interestingly, in the RNA gel blot analysis, EtBr staining of rRNAs, which was used as the loading control, showed obvious differences in the accumulation of the plastidial 16*S* rRNA (Figure 3.4.6). However, after 6 weeks growing, the amount of 16*S* rRNA accumulated to wild-type levels. The same phenomenon of reduced 16*S* rRNA accumulation was also observed in the second T-DNA line *AtRAP-2* (data not shown). As 16*S* rRNA is required for chloroplast translation these results are in agreement with reduced protein accumulation and synthesis (Figure 3.5.3, Figure 3.5.4 and Figure 3.5.5), and explain the virescent growth-retarded phenotype of *AtRAP* mutants.

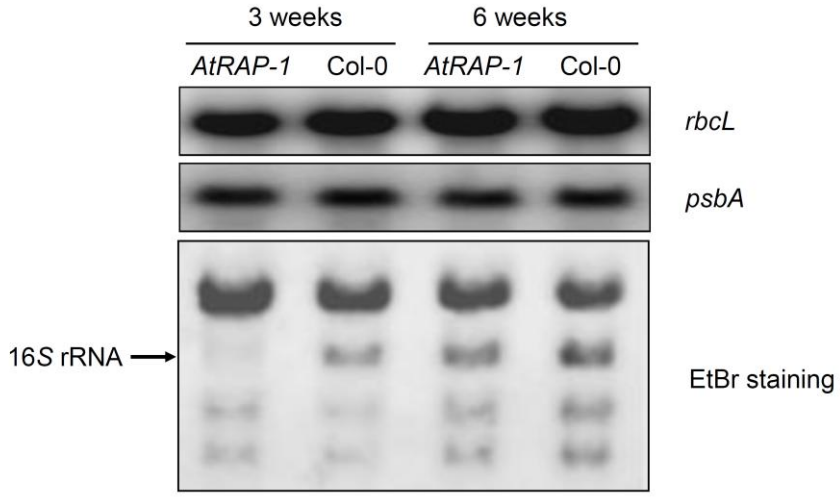


Figure 3.5.6 Accumulation of abundant chloroplast transcripts in *AtRAP-1* mutants. Total RNA was extracted from 3-week-old and 6-week-old plants (Col-0 and *AtRAP-1*) grown in the green house. 5 µg of total RNA of each line were separated on a 1% denaturing agarose gel and transferred to Hybond-N^+membranes. Probes of *psbA*, and *rbcL* generated by PCR and labeled with digoxigenin were hybridized for the determination of transcript levels. EtBr staining of the gel was used as a loading control.

3.5.4 The processing of 16*S* rRNA is affected in the *AtRAP-1* mutant

The *A. thaliana* chloroplast *rrn* operon consists of genes for 16*S* (*rrn16*), 23*S* (*rrn23*), 4.5*S* (*rrn4.5*) and 5*S* (*rrn5*) rRNAs and tRNA genes for Ile (*trnI*), Ala (*trnA*) (Figure 3.5.7 A, Leal-Klevezas et al., 2000; Strittmatter and Kössel, 1984). Processing sites within the *rrn* operon

occur at different positions, shown by vertical arrows in Figure 3.5.7 A. The processing process produces precursors of 16*S*, 5*S*, and a 23*S*-4.5*S* dicistronic intermediate, as well as tRNA precursors. In order to investigate the processing and accumulation of 16*S* rRNA in particular and of the *rrn* operon in general, different probes were generated by PCR for RNA gel blot analyses. Among them, probe A overlaps two promoter regions, PC (-141 nt) and P2 (-117 nt), as well as a processing site (-30 nt), described by Lerbs-Mache (2000); probe C binds to the region before the first processing site, detecting the 16*S* rRNA precursor; Probes B, E, D, F and G bind to 16*S* rRNA, 23*S* rRNA, 4.5*S* rRNA, and 5*S* rRNA respectively, detecting both mature rRNAs and their precursors. The processing of the 23*S* rRNA

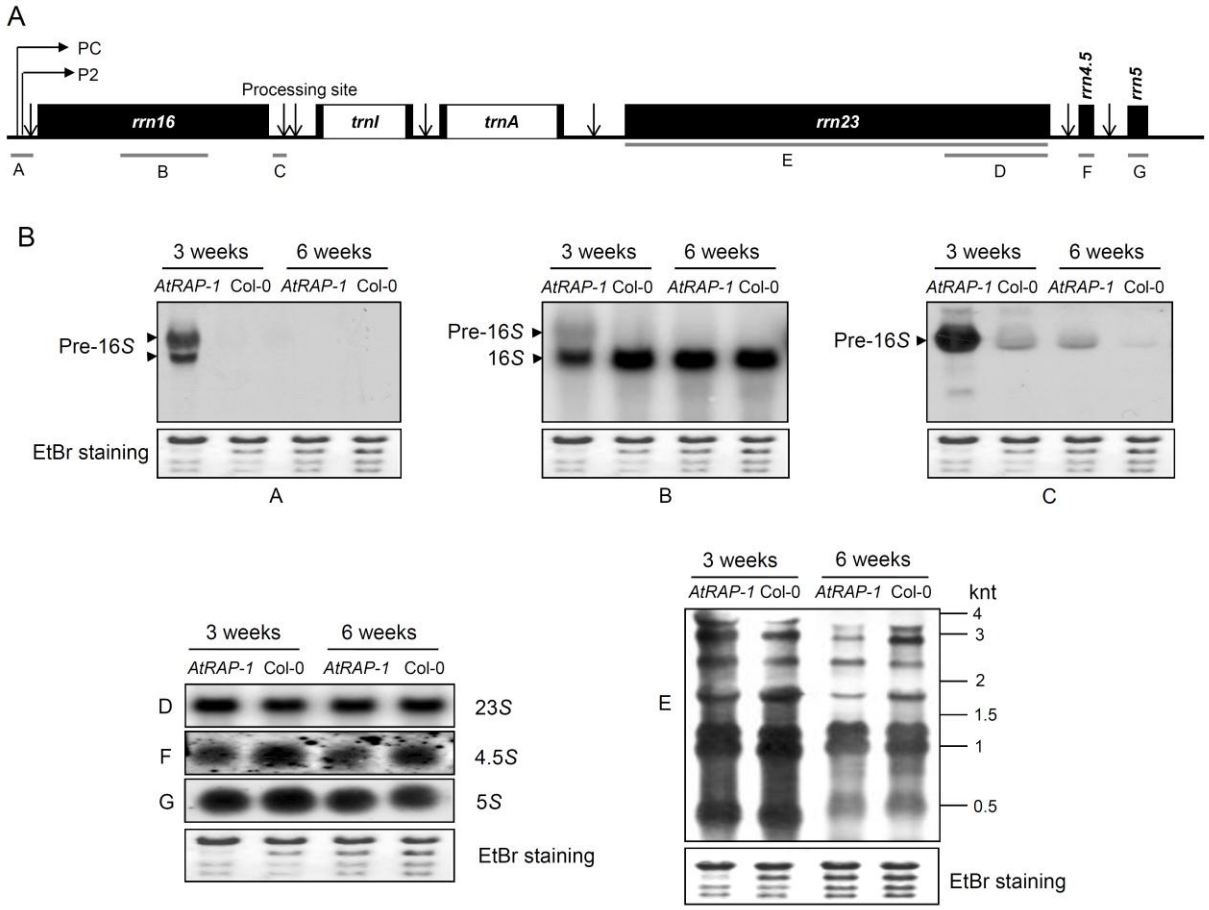


Figure 3.5.7 Accumulation of chloroplast rRNAs in wild-type and *AtRAP-1* plants.
A) The schematic representation of the *A. thaliana* chloroplast *rrn* operon is shown on the top. Black boxes indicate exons of rRNA as well as Ile-tRNA and Ala-tRNA genes. White boxes indicate introns. PC and P2 are promoters represented by curved arrows. Vertical arrows show the processing sites of the *rrn* operon. Positions of PCR-amplified probes are marked by grey lines under the operon and are indicated by letters (A-G).
B) Northern blot analysis of 3-week-old and 6-week-old plants of wild-type and *AtRAP-1* plants are shown. Total RNAs from wild-type and *AtRAP-1* mutant were separated in 1% agarose gels, transferred to nylon membranes and hybridized with different probes marked with A, B, C, D, E, F, and G, detecting precursor and mature rRNAs. EtBr-stained gels of rRNA are used as loading control.

undergoes further complicated processing events leading to at least five different products (Beligni and Mayfield, 2008, Figure 3.5.7 B, probe E). However, none of the detected processing products appeared to be altered in abundance in *AtRAP-1* as compared to the wild-type. This is also confirmed by probe D only detecting the 23*S* intermediate with a size of 1 knt. The results show that there are no changes in accumulation of most processed rRNA transcripts, except the 16*S* rRNA. The amount of mature 16*S* rRNA is clearly reduced in the 3-week-old *AtRAP-1* mutant, while a precursor form is accumulating (Figure 3.5.7, probe B). In addition, probe A detected two highly abundant precursors not seen in the wild-type which are slightly bigger than the mature 16*S* rRNA in the 3-week-old mutant plants. However, the probe C binding to the 3' end of 16*S* rRNA precursor, only detects one strong precursor. The processing of 16*S* rRNA becomes like the wild-type after 6 weeks, within which, no change was observed between the wild-type and the mutant. Taken together the RNA gel blot results, it is concluded that the 16*S* rRNA processing is affected in the early developmental stage of chloroplasts in the *AtRAP-1* mutant, and this defect can be restored after 6 weeks of growth.

4 DISCUSSION

4.1 Forward genetic approaches applied on the model organism *Chlamydomonas reinhardtii*

Recent years have seen the development of a molecular toolkit for *C. reinhardtii*. Generation of tagged insertional mutations by nuclear transformation has facilitated the rapid identification of mutant alleles (Grossman et al., 2003). Meanwhile the availability of plasmids, cosmids and bacterial artificial chromosome (BAC) libraries made the rescue of nuclear mutations possible (Lefebvre and Silflow, 1999).

This study focused on the identification of mutated genes which were generated by random insertion of antibiotic resistance markers into the nuclear genome of *C. reinhardtii*. At this, for 5 out of 16 provided mutants possessing a PSII phenotype the corresponding gene could be identified (section 3.2). However, even though the mutagenesis methods and downstream applications for gene identifications have clearly been improved during the last years, the identification of the nuclear mutation responsible for the PSII phenotype was not always possible. In some cases, it was observed that the antibiotic resistance marker was not linked to the PSII phenotype or the marker could not be detected in the mutant genome at all (table 3.2.1). Main reasons for that were multiple insertions of the mutagenic agent, insertions of truncated markers, and deletions or rearrangements occurring during the transformation procedure which hamper or obviate the application of techniques used. However, as in *C. reinhardtii*, gene-targeted mutagenesis, e.g. by homologous recombination, is not possible to date, random insertional mutagenesis in combination with PCR based downstream applications for the identification of the disrupted genes is still a powerful tool to elucidate protein functions.

Classic forward genetic approaches for gene cloning include plasmid rescue and map-based techniques, where the latter technique worked successfully for the identification of a disruption of the *OHP2* gene in the *101a* mutant (section 3.3.1). A modified PCR based approach, “inverse PCR”, was effectively used for several PSII mutants and identified mutations in genes encoding a putative transcription factor (mutant *45a*), a speract/scavenger receptor domain protein (mutant *42b*), as well as CLR24, a member of the OPR repeat protein family (mutant *101b*, section 3.2.3). In order to rescue identified mutations, two independent complementation methods were used. The transformation of *OHP2* cDNA under control of the *PsaD* promoter together with paromomycin resistance gene (*aphVIII*) successfully

complemented the *101a* (*ohp2*) phenotype. The cDNA complementation used provides an additional selection method for positive transformants based on an introduced resistance gene besides phenotype rescue. However, the *C. reinhardtii* genome exhibiting a high GC rich content often causes difficulties to amplify the full length cDNA. In a second approach, the transformation of a BAC clone containing the *CLR24* genomic sequence restored the CLR24 function in the *101b* (*clr24*) mutant. Nevertheless, the usage of BAC clones requires the availability of a BAC library which offers a complete coverage of the *C. reinhardtii* genome. A second disadvantage is a lack of eukaryotic markers which allow a selection for positive transformants if the restoration of the phenotype is not immediately seen. Nowadays, some novel shuttle markers, like *aadA* and *aphVIII*, were used for modifying BACs with antibiotic resistances, which allow a broader usage of BAC clone transformation (Meslet-Cladiere and Vallon, 2011).

In order to further optimize the described forward genetic approach for *C. reinhardtii* in the future, great efforts are needed for (I) more efficient transformation methods to avoid multiple insertion, or insertion of truncated vectors (II) optimized map-based cloning, with more molecular markers placed on the linkage map and higher quality sequence information in online databases, which still have vacuities on the mating type and highly repetitive regions; (III) BAC library covering complete *C. reinhardtii* genome and modified BAC DNA to facilitate the screening of transformants. Although online databases provide more accurate *C. reinhardtii* genome information, which also make reverse genetic approaches, like antisense and RNA interference technologies, possible, the numerous advantages of forward genetic approaches cannot be overlooked and will provide itself a bright future.

The following chapters will focus on a detailed discussion of the *ohp2* and *clr24* mutants as well as the functions of the proteins encoded by the disrupted genes identified in course of the forward genetic screen used in this study.

4.2 The *Chlamydomonas reinhardtii* OHP2 protein is involved in the accumulation of the PSII reaction center protein D1

By applying a mapped-based strategy, the *OHP2* gene was found to cause the PSII phenotype in the mutant *101a* (section 3.3.1). Subsequent molecular and biochemical analyses revealed the OHP2 protein to be specifically involved in the accumulation of the D1 protein (section 3.3.4). At this, normal *psbA* mRNA levels but no detectable D1 protein synthesis in pulse labeling experiments suggested a role of OHP2 either in the translation process of the *psbA* mRNA or in stabilization of the D1 protein (sections 3.1, section 3.3.4).

The OHP2 protein from *C. reinhardtii* belongs to the early light induced proteins (Elips) super protein family, which includes distant relatives of LHC protein family with conserved chlorophyll binding residues (Adamska et al., 2001). A multiple sequence alignment of OHP2 homologues from various photosynthetic organisms ranging from cyanobacteria to land plants shows a high conservation of the primary protein structure (Figure 3.3.5). This suggests that the one-helix Elip present in ancient cyanobacteria might be a progenitor of plants and algal antenna proteins. Since the more recently evolved antenna LHC proteins function as the light harvesting system, the Elips might have different functions (Montane and Kloppstech, 2000). The *A. thaliana* OHP2 protein has already been characterized. It is present in thylakoid membranes with induced accumulation by light stress of both *OHP2* transcript and protein levels (Andersson et al., 2003). Its co-localization with PSI and sensitivity to light stress proposed that OHP2 protein could prevent or lower light stress-induced damage, and help PSI to be resistant to photoinhibition. Another hypothesis about Elip's functions suggested that they might also protect PSII under light stress via binding free chlorophyll molecules and preventing formation of free radicals and/or stocking excitation energy (Montane and Kloppstech, 2000).

In contrast to what was reported for *A. thaliana*, this study does not provide any evidence for OHP2 from *C. reinhardtii* to be associated with PSI complexes. Considering the putative chlorophyll binding activity of OHP2 as indicated by the occurrence of many highly conserved amino acid residues within the proposed chlorophyll binding region (Figure 3.3.4) and its specific effect on the PSII protein D1, several functions of OHP2 are imaginable. In barley, it was described that chlorophyll regulates the accumulation of the D1 protein by increasing the protein stability (Kim et al., 1994). It was additionally proposed that the binding of co-factors to D1 is facilitated by an observed pausing of ribosomes at specific sites during translation of membrane-bound *psbA* mRNA. Furthermore, He and Vermaas (1998) could show that chlorophyll *a* availability controls D1 biosynthesis and D1 precursor processing in Synechocystis. As D1 is described to be co-translationally inserted into the thylakoid membrane (Zhang et al., 1999, 2000), and OHP2 is a membrane localized protein (Irene Meindl, unpublished data), these data indicate that OHP2 in *C. reinhardtii* as a putative chlorophyll carrier protein might be involved in the transfer of chlorophyll to newly synthesized or nascent D1 proteins (Figure 4.1). The possible lack of chlorophyll incorporation into D1 in the *ohp2* mutant might affect D1 synthesis directly or destabilize readily synthesized D1 proteins. Taking into account that Mullet et al. (1990) described a co-translational binding of chlorophyll *a* produced in illuminated plants to stabilize D1 nascent

polypeptides, the above hypothesized role of OHP2 during D1 synthesis seems to be more likely.

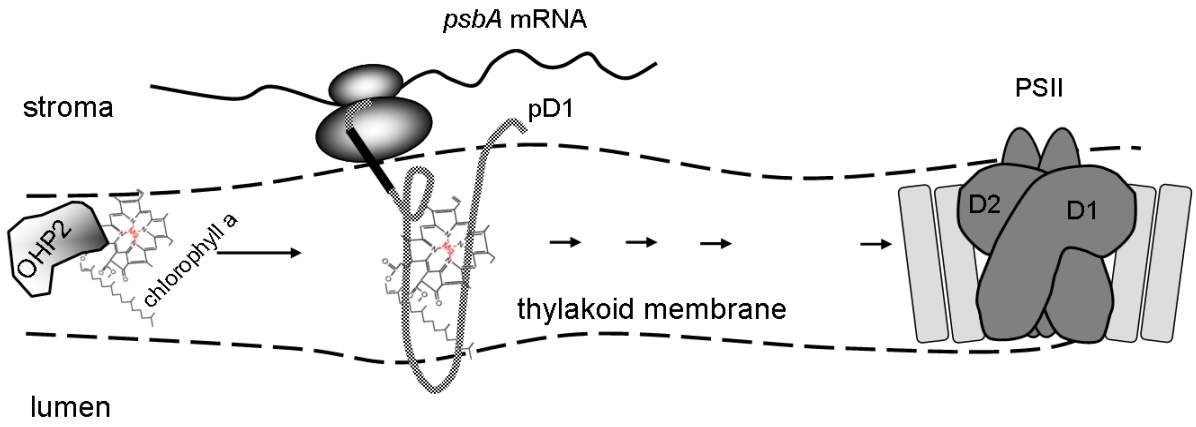


Figure 4.1 Hypothesized working model for the *C. reinhardtii* OHP2 protein

The membrane located OHP2 protein could bind to chlorophyll *a* and transfer it to the nascent D1 protein by which it is stabilized.

The specific effect of OHP2 on D1 synthesis seems surprising, as many other thylakoid membrane proteins require chlorophyll co-factors, but it might be explained by the extremely high turnover rates of the D1 protein. Particularly under higher light conditions the D1 protein, but not other photosystem subunits, is photo-damaged and needs to be replaced by the so-called repair cycle (Kato and Sakamoto, 2009). However, a direct interaction of OHP2 and the D1 protein in *C. reinhardtii* as well as the predicted chlorophyll binding activity of OHP2 remains to be shown. Also, a possible protective function of OHP2 for PSII during light stress has to be elucidated in the future.

Another potential function of OHP2 in *psbA* translation, e.g. as a component of the translation initiation complex and/or *psbA* mRNA binding protein (compare section 1.3.1.2.2), can also not be excluded but is highly unlikely, considering the relation to chlorophyll binding proteins and a lack of putative RNA binding domains within this small 11 kDa-protein. However, e. g. polysome loading experiments and RNA binding assays would be required to exclude this possibility.

4.3 The *Chlamydomonas reinhardtii* CLR24 protein is involved in PSII dimer formation

The second *C. reinhardtii* mutant investigated in more detail in course of this study, revealed an insertion in the *CLR24* gene encoding a member of the OPR repeat protein family (section 3.4). Phenotypical and biochemical analyses of the *clr24* mutant showed that the PSII activity is affected which is likely to be caused by the inability of PSII dimer formation (section 3.4.3). By detecting transcript accumulations of several chloroplast-encoded low molecular weight PSII subunits, the mature *psbI* mRNA was found to be missing in the *clr24* mutant (section 3.4.4.1, Figure 3.4.6).

Unlike vascular plants, for which most chloroplast genes are organized into polycistronic transcription units, most of the transcripts in *C. reinhardtii* are transcribed as monocistronic. However, the *atpA* gene cluster including the affected *psbI* gene is one of the special cases including four co-transcribed genes. One gene expression working model for the *atpA* cluster was set up in a previous study, which involves three sites of transcription initiation, up to three sites of transcript 5' processing and four sites of transcription termination or 3' processing, indicated by deletion mutants and mapping experiments (Drapier et al., 1998).

Detailed Northern blot experiments of polycistronic *atpA-psbI-cemA-atpH* transcripts verified that the processing of the entire transcript is affected in the *clr24* mutant, which leads to an increased accumulation of mature monocistronic *atpA* and *atpH*, and undetectable amounts of mature monocistronic *psbI*, tricistronic *psbI-cemA-atpH* as well as dicistronic *psbI-cemA*, which is the only mature form of *cemA* mRNA (section 3.4.4.2, Figure 3.4.7). All informations obtained, point to an affected PSII activity caused by altered processing of the *atpA-psbI-cemA-atpH* polycistronic transcript, more precisely a lack of *psbI* and/or *cemA* messages.

In *C. reinhardtii* $\triangle$*psbI* mutants as well as nuclear mutants, which were found to lack *cemA* transcripts via forward genetic screening have been described. Whereas in higher plants, *psbI* is co-transcribed with *psbK*, in *C. reinhardtii* it is located downstream of *atpA* (Figure 3.4.7 A). As seen for the *clr24* mutant (Figure 3.1.1, Table 3.2.1), the *C. reinhardtii* $\triangle$*psbI* mutant in which the *psbI* gene was deleted by an *aadA* cassette reveals a decreased accumulation of PSII subunits and accordingly, reduced PSII activity as indicated by chlorophyll fluorescence measurements (Künstner et al., 1995). Additionally, the $\triangle$*psbI* mutant revealed a slight growth under photoautotrophic conditions in lower light (less than 100 $\mu E/m^2/s$) which was also observed for the *clr24* mutant (Figure 3.4.2), but not under high light (600 $\mu E/m^2/s$),

demonstrating its light sensitive phenotype. Unfortunately, there is no further characterization of this ⊿*psbI* mutant in *C. reinhardtii* to clarify its precise function for maintaining PSII performance. However, tobacco ⊿*psbI* mutants obtained by a transplastomic knock-out approach also can grow photoautotrophically and are sensitive to high light. Here it was described that, in correlation with the observed phenotype in *clr24* (section 3.4.3), PsbI is required for PSII dimer stabilization. Additionally, PsbI was postulated to be involved in forward electron transport, as well as efficient phosphorylation of PSII-RC proteins. The final conclusion focuses on the point that PsbI is required for the stability of higher order complexes of PSII and proper functions of PSII in higher plants (Schwenkert et al., 2006). The latest study on PsbI was performed in thermophilic cyanobacteria (Kawakami et al., 2011a). The PSII dimer stabilization was also found to be affected in cyanobacteria ⊿*psbI* mutants, but the difference between the wild-type and mutant is less severe than in *C. reinhardtii* and higher plants. One of the explanations is that there exists a more sophisticated collaboration among various subunits during PSII dimer formation in eukaryotes, which leads to mechanisms that are more complex and guarantee a higher level of regulation between the chloroplast and the nucleus.

Taken together, the data suggest the missing *psbI* mRNA in the *clr24* mutant to be responsible for the observed effect on PSII dimer formation. However, it cannot be totally excluded that there are still trace amounts of PsbI protein translated from polycistronic *atpA-psbI-cemA* and *atpA-psbI-cemA-atpH* transcripts, since no evidence showed the PsbI protein only to be transcribed from monocistronic transcripts. Nevertheless, the post-transcriptional regulation of *psbI* mRNA is affected in the *clr24* mutant, which is likely to be the direct cause of reduced PSII dimer formation and PSII activity. In addition, more precise growth test of the *clr24* mutant under stress light condition and investigations of the electron transport ability should be in the work plan, in order to better understand the phenotype of the *clr24* mutant as well as the function of *C. reinhardtii* PsbI protein.

However, the *clr24* mutant also revealed a lack of *cemA* mRNA. The CemA (Chloroplast Envelop Membrane) protein, which was also named Ycf10 (hypothetical chloroplast open reading frame) due to its immuno-localization in the inner membrane of the pea chloroplast envelopes, is highly conserved in photosynthetic organisms (Price et al., 1995). The function of CemA was mainly analyzed in cyanobacteria and *C. reinhardtii*. ⊿*cemA* mutants of both organisms show similar phenotypes, which indicate the possibility that CemA may have a role in CO_2 assimilation, whereas it is independent of photosynthesis. A light sensitive phenotype was also observed for the mutant, therefore, CemA is suggested to be involved in a process

essential for adaptation of cells to high photosynthetic growth rate (Rolland et al., 1997). It is worth to mention that there still might be functional CemA protein in the *clr24* mutant, since there is no evidence for CemA protein to only be translated from the dicistronic transcript.

According to these data, it is unlikely that the lack of *cemA* mRNA in *clr24* has a direct impact on the PSII complex formation and photosynthetic performance. However, the light-sensitivity of the *clr24* mutant might at least partially be caused by missing *cemA* mRNA. Given all the information above, the ability of CO_2 assimilation, especially under photoinhibitory condition should be analyzed in the following work in order to better understand the phenotype of the *clr24* mutant, as well as the translation mechanism of *cemA* mRNA.

Besides *psbI* and *cemA*, altered transcript accumulations between wild-type and mutants were also observed for *atpA* and *atpH*, which encode the ATPase α or CFo-III subunit, respectively. In the *clr24* mutant, the accumulation of monocistronic *atpA* and *atpH* transcripts is increased (Figure 3.4.7 B). Previous investigations showed that the translation of the ATPase α subunit is not significantly affected by altered levels of *atpA* transcripts (Drapier et al., 1992), therefore the unusual *atpA* accumulation could be a secondary effect, which does not affect the protein level of ATPase in the *clr24* mutant. The function of AtpH in *C. reinhardtii* was characterized with the *ac46* mutant, which is lacking monocistronic *atpH* mRNA, and the mutant is highly sensitive to light stress, but grows photoautotrophically in the low light as wild-type cells (Majeran et al., 2001). However, there is no study about the correlation between AtpH protein levels and the available amount of *atpH* transcripts. It is unclear if the accumulation of AtpH protein is also affected in *clr24* mutant.

The increased accumulation of mature *atpA* and *atpH* transcripts in *clr24* might be explained by higher transcriptional rates trying to compensate for the lack of mature *psbI* and *psbI-cemA* mRNAs. This might be indicated by a slightly increased accumulation of the *atpA-psbI-cemA-atpH* polycistronic transcript. However, the increase of this message is less than would be expected from the increase of *atpA* and *atpH* transcripts, which might indicate also a destabilization of the polycistronic transcript or an altered usage of promoters within this gene cluster. Taking *atpH* as an example, the monocistronic *atpH* might be produced either by transcription from its own promoter, or by processing from longer transcripts initiated from *atpA* or *psbI* proximal promoters. The ratio of the *atpH* transcripts obtained from these two processes might be altered in the *clr24* mutant, which needs further analysis. Also additional regulatory mechanisms involving transcript stabilizations cannot be excluded. The

investigation of transcriptional rates from each of the three promoters described could shed more light on this phenomenon.

Previous research suggests that RNA-binding proteins, as well as RNA stem-loop structures play a pivotal role in determining transcript abundance (Herrin and Nickelsen, 2004; Rott et al., 1998). Interestingly, OPR proteins are predicted to have RNA binding activity and are described to be involved in RNA processing and stabilization processes (compare section 1.3.2.3). Similar the OPR protein CLR24 might be involved in the posttranscriptional regulation of the *atpA-psbI-cemA-atpH* transcript, via direct or indirect interaction. According to the Northern results in Figure 3.4.7, most strikingly all transcripts which are initiated or processed upstream of *psbI* and therefore reveal the same 5' end are not detectable in the *clr24* mutant. This suggests a role of CLR24 in stabilization or processing of these transcripts by binding to their 5'regions (Figure 4.2). Interestingly a deletion of this region, including the

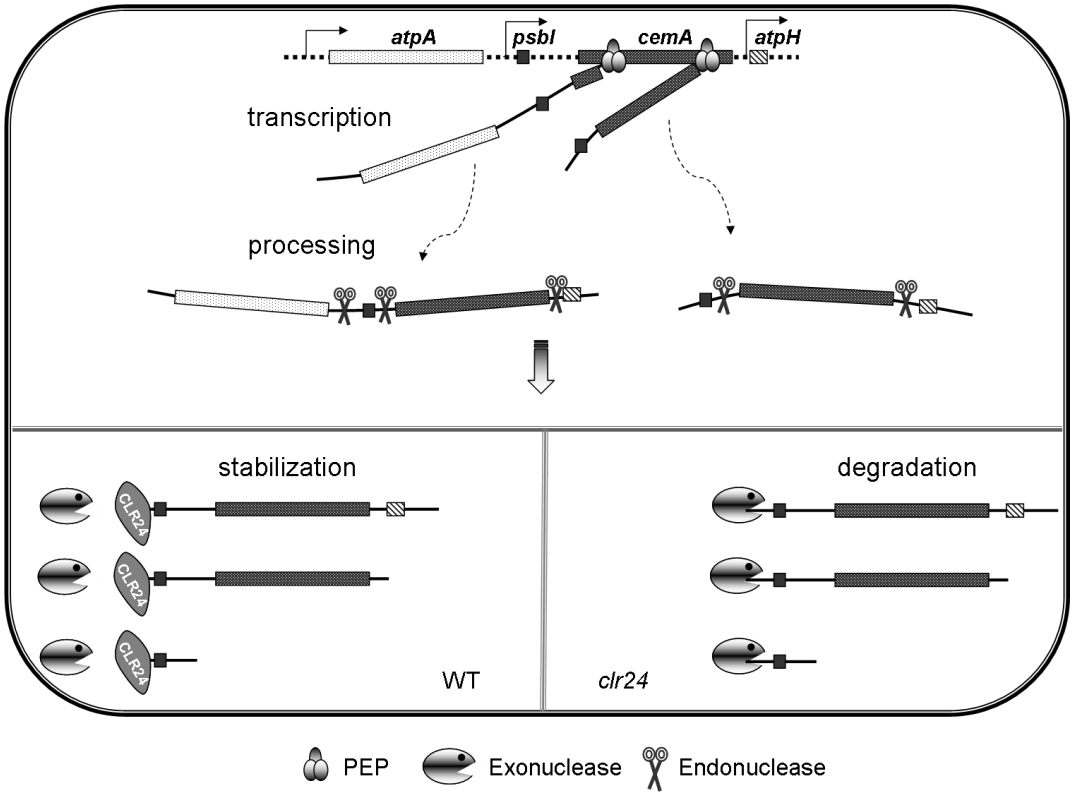


Figure 4.2 Hypothesized working model for the *C. reinhardtii* CLR24 protein as an RNA stability factor. The *atpA* gene cluster is shown on the top with co-transcribed *atpA*, *psbI*, *cemA* and *atpH* messages. The three promoters are indicated by bent arrows. The CLR24 protein could interact with 5' termini of *psbI-cemA-atpH*, *psbI-cemA*, and *psbI* transcripts, in order to protect them from degradation by exonucleases. In the *clr24* mutant background, the absence of CLR24 leads instability of transcripts revealing *psbI* at their 5'ends.

psbI promoter and part of the *psbI* 5'UTR also leads to missing mature *psbI* transcripts (Drapier et al., 1992). This effect might clearly be influenced by missing regulatory cis-elements, but might also indicate a deletion of the possible CLR24 binding site. These possibilities will be verified by further RNA binding experiments confirming the RNA binding capacity of CLR24 and by precise mapping of its binding site within the transcripts initiated or processed immediately upstream of the *psbI* message.

Taken together, the data obtained in course of this study indicate a role of CLR24 as mRNA stabilization/processing factor required for the accumulation of all the three transcripts possessing *psbI* at 5' termini. The absence of CLR24 causes the loss of mature *psbI* mRNA and most likely the loss of PsbI proteins leading to the effects observed in PSII dimer formation and reduced photosynthetic activity.

4.4 The *Arabidopsis thaliana* AtRAP protein is involved in chloroplast 16*S* rRNA processing

A single OPR protein, AtRAP, was identified in *A. thaliana* (section 3.5). Considering the important role of OPR protein functions in chloroplast RNA metabolism described previously and in this study (compare section 1.3.2.3 and 4.3), the elucidation of AtRAP's function in *A. thaliana* was of particular interest also from an evolutionary point of view.

AtRAP T-DNA insertion lines revealed growth retardation, a pale green phenotype, and reduced photosynthetic activity (section 3.5.2). Furthermore, the mutants exhibited normal levels of abundant chloroplast transcripts (Figure 3.5.6), whereas their translation and therefore accumulation of chloroplast encoded proteins was dramatically reduced in early growth stages (Figures 3.5.3 and 3.5.5). The observed translation defect of the chloroplast machinery is verified to correlate with unusual ribosome RNA processing. To be precise, the maturation of 16*S* rRNA is severly affected. While decreased levels of mature 16*S* rRNA were detected in *AtRAP* T-DNA lines, a larger precursor accumulated as compared to the wild-type (section 3.5.4, Figure 3.5.7). In terms of that, it is clearly indicated that the phenotype of *AtRAP* mutants described above is specifically caused by inefficient processing of the chloroplastic16*S* rRNA.

4.4.1 Organisation and processing of the ribosomal RNA gene cluster in chloroplasts

Similar to its eubacterial progenitor, chloroplast ribosomes are composed of two subunits, 50*S* and 30*S*, which together form the translation machinery, the 70*S* ribosomes (Harris et al.,

1994). Ribosomal subunits comprise more than 50 ribosomal proteins, together with four ribosomal RNAs, which are encoded in one gene cluster, functioning with the catalytic activity of the ribosome (Nissen et al., 2000). The 23*S* rRNAs are split into 23*S* and 4.5*S* in chloroplasts (compare Figure 3.5.7), and the latter shares high homology with the 3' end of the bacterial 23*S*. Furthermore, the chloroplast 16*S* and 23*S* rRNAs are flanked by tRNAs specific for isoleucine and alanine, while a tRNA for arginine is encoded downstream of the 5*S* rRNA (Strittmatter and Kössel, 1984). Unlike the multiple copy of rRNA operon in bacteria, there are only two rRNA operons in the chloroplast genome, which are located in the inverted repeats. However, in spite of vast evolutionary distance, the chloroplast rRNA gene cluster still resembles that of bacteria in terms of organization of coding sequences and co-transcription of genes.

Maturation of rRNA precursors performed by endo- and exoribonucleases, in a ribosome assembly-assisted manner, is an essential step for ribosome function. To date, most of the investigations on rRNA maturation mechanism are based on *E. coli*, which showed that RNase III, in concert with RNase E and G, is responsible for the endonucleolytic cleavages, while RNaseT performs the exonucleolytic 3' trimming step (Davies et al., 2010). Some higher plant mutants with defects in ribosomal RNA processing were also under characterization, however, the precise rRNA processing mechanisms are still not clear. Ribonucleases were first considered as cleaving enzymes without sequence specificity (Stoppel and Meurer, 2012). However nowadays, increasing ribonucleases have been verified to display specificity for sequences or structures, which help regulate the transcript abundance according to environment changes (Pfalz et al., 2009; Stoppel et al., 2011). The precise regulatory role for ribonucleases has yet to be convincingly shown. It was also clarified that multiple ribonucleases are involved in the rRNA maturation process with overlapping functions and specificities. Several nucleus-encoded proteins, such as PPRs have been hypothesized to form high-molecular-weight complexes with ribonucleases (Stern et al., 2010). Several nucleus encoded proteins have been described to be involved in chloroplast rRNA processing. Among the already known factors, only a few of them have predicted ribonuclease activity, and all of them are lacking clear mechanism explanations (Table 4.1; Bollenbach et al., 2005; Cheng and Deutscher, 2005; Kishine et al., 2004; Walter et al., 2002). The lack of several known factors leads to general defects in chloroplast rRNA processing, as described for RNR1, BPG2 and DCL, while other proteins have their specific targets, for instance, DCL is required for 23*S*-4.5*S* RNA processing, CSP41a & CSP41b for 23*S* rRNA processing, and PRBP for 23*S* rRNA maturation (compare table 4.1). The absence of these

proteins affects chloroplast development to various extents, for example with or without influence on chloroplast mRNA accumulation. There are more than 180 ribonucleases annotated in *A. thaliana*, but only 17 are predicted to localize in the chloroplast, which are also approved by biochemical analyses (Arraiano et al., 2010). All the information above suggests that during evolution from prokaryotes to higher plants, the ribonucleases obtained diverse functions, which make the rRNA processing in chloroplasts more complex to adapt to environmental changes. Probably the rRNA processing in chloroplasts requires different factors to co-operate together.

Table 4.1 Characterized nucleus-encoded proteins involved in chloroplast ribosomal RNA processing

Higher plants protein	Mutant phenotype	Function	Reference
WCO (Arabidopsis)	albino cotyledons, green leaves; low levels of chloroplast mRNA, normal nuclear encoded mRNAs	maturation of 16*S* rRNA	Yamamoto et al., 2000
HCF7 (Maize)	yellow leaves, retarded growth; normal accumulation of chloroplast and nuclear encoded mRNAs	maturation of 16*S* rRNA	Barkan, 1993
PNPase (Arabidopsis)	-	metabolism of all major classes of plastid RNAs	Walter et al., 2002
BPG2 (Arabidopsis)	retarded growth; normal accumulation of chloroplast encoded mRNAs; abnormal accumulation of chloroplast rRNAs	maturation of all chloroplast rRNAs	Komatsu et al., 2010
RNR1 (Arabidopsis)	white cotyledons, pale green leaves; normal accumulation of chloroplast encoded mRNAs	3'–5' exoribonuclease involved in the maturation of 23*S*, 16*S* and 5*S* rRNAs	Bollenbach et al., 2005
DAL(Arabidopsis)	yellow leaves; low accumulation of nuclear and chloroplast encoded mRNAs	maturation of all chloroplast rRNA	Bisanz et al., 2003
DCL(Tomato)	defective chloroplast and leaf; normal accumulation of chloroplast and nuclear encoded mRNAs	plastid ribosome assembly; 23*S*-4.5*S* rRNA processing	Bellaoui et al., 2003
PRBP (Tobacco)	yellow leaves; normal accumulation of chloroplast and nuclear encoded mRNAs	maturation of 4.5*S* rRNA	Park et al., 2011
CSP41a & b (Arabidopsis)	double mutant is lethal, mutants with no CSP41b and greatly reduced levels of CSP41a show retardate growth; normal accumulation of other chloroplast rRNAs and mRNAs	metabolism of 23*S* rRNA	Beligni and Mayfield, 2008

There are several proteins (table 4.1) whose absence leads to specific processing defects on 16*S* rRNA, which are similar to the phenotype observed for *AtRAP*. A *A. thaliana* mutant lacking the WCO (WHITECOTYLEDONS) protein could not grow photoautotrophically in the early growing stage and showed an inefficient processing of 16*S* rRNA (Yamamoto et al., 2000). However, in later stages the mutant's phenotype turns to be like the wild-type. In addition, the absence of WCO also causes reduced transcription levels of chloroplast encoded genes, which is not observed in the *AtRAP* mutant (Figure 3.5.6). Another protein is HCF7 in maize, which is one of the earliest characterized proteins involved in ribosomal RNA processing. The absence of HCF7 protein in maize leads to a similar phenotype as seen for

AtRAP, such as retarded growth and reduced accumulation of chloroplast encoded proteins while mRNA levels remained unaltered. It is hypothesized that WCO is a counterpart of HCF7 in *A. thaliana* due to their similar function (Yamamoto et al., 2000). This assumption could be supplemented by AtRAP. However, the gene causing the mutation in WCO is not identified yet, but was approximately mapped to the top of chromosome 1. According to that, the mutation in *WCO* is not identical with *AtRAP*, as this gene is located to chromosome 2. Due to almost identical phenotypes of *WCO* and *AtRAP*, one might assume though, that the protein causing the *WCO* phenotype and AtRAP act together in the described16*S* processing events. Once the gene in *WCO* is identified, it would be interesting to investigate a potential interaction of these proteins.

4.4.2 How is AtRAP involved in chloroplast 16*S* rRNA maturation?

The processing sites for cleavage of pre-16*S* rRNA from the 7.4 kb rRNA operon have already been characterized by 5’ end and 3’ end mapping in *A. thaliana*, but the involved endonuclease(s) are unknown to date (Stoppel and Meurer, 2012, Figure 4.3). Northern blot results in the present work show this primary cleavage is normal in *AtRAP* mutants, which accumulate certain amount of pre-16*S* rRNA, followed by an abnormal maturation process via endonucleases on the 5’ end and potentially exonucleolytic 3’ end trimming. Several 3’ to 5’ exoribonucleases have been reported for rRNA processing, such as RNR1 and PNPase, but no 5’ to 3’ endonuclease has been characterized (Bollenbach et al., 2005; Yehudai-Resheff et al., 2001).

Presented Northern blot data with different probes suggest that the maturation of pre-16*S* rRNA might be affected at both 5’ end and 3’ end in the *AtRAP-1* mutant. Based on the working mechanisms of rRNA processing in bacteria, there could be several hypotheses for the function of AtRAP: (1) AtRAP is a ribonuclease by itself, working on 5’ and 3’ end of pre-16*S* RNA, in this case, AtRAP is supposed to have nuclease activity as well as specific RNA targeting function; (2) AtRAP, which is predicted to have RNA binding ability could assist or facilitate the targeting of certain ribonucleases to pre-16*S* rRNA to fulfill the processing; (3) AtRAP is required for ribosome assembly, which has already been proved being necessary for rRNA maturation (Holloway and Herrin, 1998).

The first hypothesis is supported by the occurrence of a RAP domain at the C-terminus of the AtRAP protein. This domain is also designated as restriction_endonuclease_like region (DUF559) and described in many bacterial endonucleases. However, if this domain has indeed endonucleolytic activity in AtRAP remains to be shown.

The second possibility is supported by recent primer extention analyses which revealed an accumulation of 16*S* premature transcripts starting at P2 and the 5'end processing site as well as an accumulation of transcript ends between P2 and the mature transcript, which have not been described yet but are also detected to a lesser extent in the wild-type (Laura Kleinknecht, unpublished data). These results indicate that the processing occurs in the *AtRAP-1* mutant, but less efficiently. Furthermore, recently small RNAs (16-28 nt non-coding RNA) located in chloroplast genome were indicated to be footprints of RNA-binding proteins, such as PPR proteins, playing roles during RNA maturation processes (Ruwe and Schmitz-Linneweber, 2012). This study also revealed several small RNAs in the region of the 16*S* rRNA. Using these footprints, a comparison of nuclease-protected regions within the 16*S* rRNA region in the *AtRAP* mutant and the wild-type indicate that the binding site of AtRAP is at the 5'end of the premature 16*S* rRNA sequence around P2 promoter (Laura Kleinknecht, unpublished data).

However, as processing of the 16*S* pre-transcript still occurs, it is unlikely that the ribonucleolytic activity itself is missing in the *AtRAP* mutant which favors the second hypothesis over the first one. There are also no evidences to support the third hypothesis. Further experiments including a detailed analysis of ribosome assembly in *AtRAP* mutants would be required, by which a potential function of AtRAP in efficient ribosome assembly could be clarified.

Taken together, these data indicate that AtRAP might guide ribonucleases to their site of action at the 5'end of the premature 16*S* rRNA or increases their efficiency especially in early growth stages when the chloroplasts need high translation levels for development (Figure 4.3). One might speculate that binding of AtRAP to the 5'end of the 16*S* rRNA precursor changes its secondary structure which facilitates the binding of proteins involved in the nucleolytic events, at this enhancing the efficiency of 16*S* maturation. In contrast, in the *AtRAP* mutant the processing events are less efficient and 16*S* precursors accumulate (Figure 4.3). The affected processing of 16*S* rRNA causes inefficient ribosome assembly and therefore inefficient chloroplast translation, which explains the mutant's phenotype.

As mentioned above, the deficient phenotype of AtRAP is only present in the early stage of plant development, which is also observed for other 16*S* processing mutants, such as the *wco* mutant. One explanation is that the requirement of AtRAP changes during plant development. It might be expressed at high levels only in the early chloroplast development to increase the assembly of functional ribosomes or its function might be taken over by other proteins. Another explanation is that the mature 16*S* rRNA is highly stable and accumulates to

sufficient amounts during development which makes AtRAP more dispensable in later stages (Harris et al., 1994).

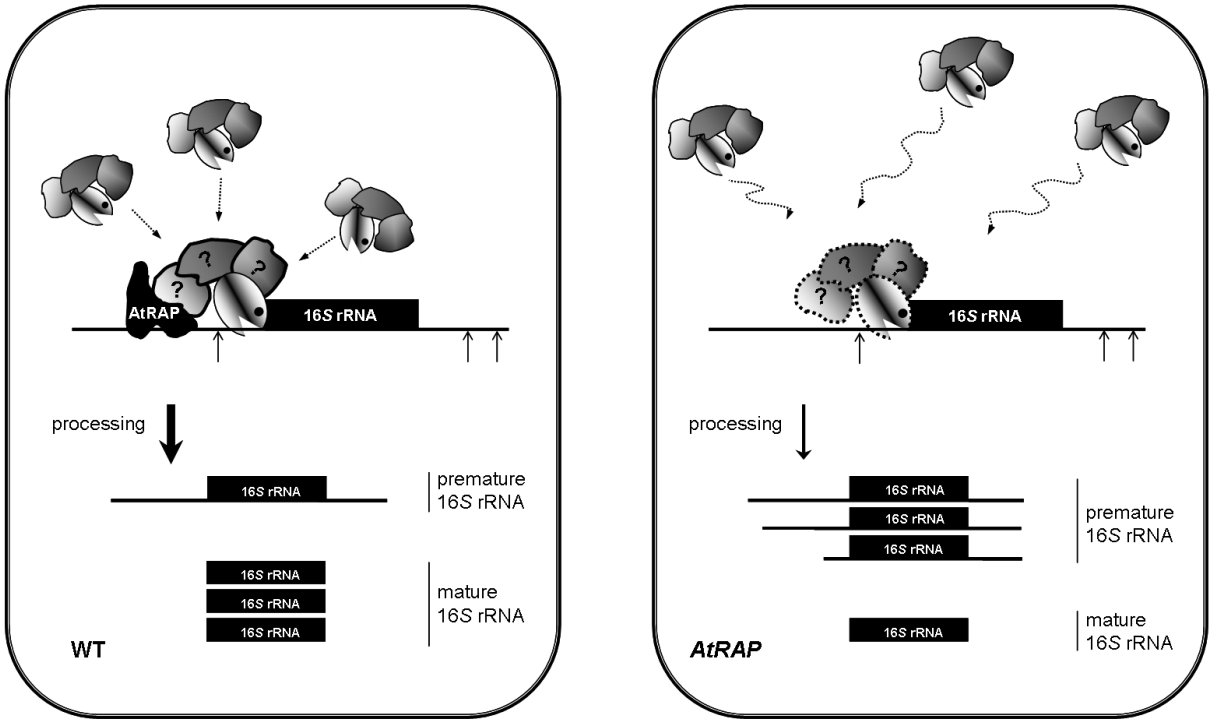


Figure 4.3 Hypothesized working model for the AtRAP protein. The map of pre-16*S* rRNA is shown in the figure, with processing site depicted as straight arrows. In the wild-type (left), AtRAP could assist a processosome consisting of certain ribonucleases and potential auxiliary factors to bind to premature 16*S* rRNA and cleavage the precursor at processing site. The processing becomes inefficient in the *AtRAP* mutant (right), as indicated by curved arrows and dashed outlines, which leads to the accumulation of premature 16*S* rRNAs.

This hypothesis needs confirmation by further experiments, such as an analysis of the expression levels of AtRAP transcripts during plant development, detailed RNA protection experiments, as well as *in vivo* and *in vitro* RNA-binding experiments. At this, it would be interesting to determine the functions of the proposed RNA-binding domains of AtRAP, both the RAP domain and the OPR repeats. Furthermore, the generation of specific antibodies against AtRAP and other proteins involved in 16*S* rRNA processing could identify possible interaction partners and elucidate potentially formed higher molecular weight complexes which may be acting as a "processosome" for the maturation of this important chloroplast rRNA.

4.4.3 AtRAP - a broader view

The OPR family consists of more than 100 members in the green alga *C. reinhardtii*, whereas in higher plants, like *A. thaliana*, only a single OPR protein could be identified (compare section 1.3.2.3). This points to a slow expansion of this protein family in higher plants, in contrast to Chlorophytes. However, the appearance is reversed looking at other repeat proteins in these groups: The PPR proteins, which have also been described to play important roles in organellar RNA metabolism exhibit more than 450 members in higher plants, whereas the nuclear genome of *C. reinhardtii* encodes only 11 PPR proteins (section 1.3.2.2). It might therefore be assumed that in higher plants PPR proteins take over the functions fulfilled by OPR proteins in Chlorophyta.

Having a closer look at the single OPR encoded by nuclear genome of diverse Streptophyta, it is indicated that all these proteins have the same evolutionary origin. As shown in an alignment of selected OPR representatives from higher plants all these proteins reveal a high conservation, especially at the C-termini containing the predicted RAP domain/ Restriction_endonuclease_like region (Figure 4.3). At this, it is tempting to speculate that these OPR proteins have a similar function in 16*S* rRNA metabolism described here for AtRAP. However, if and how these OPR proteins in other organisms than *A. thaliana* are involved in this process remains to be elucidated.

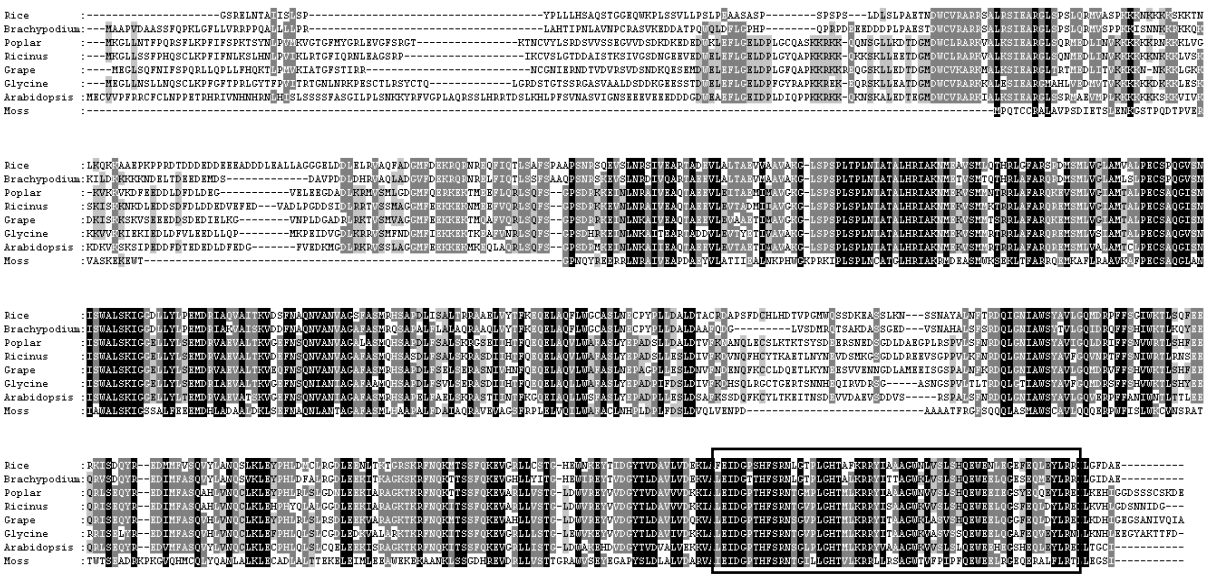


Figure 4.3 Sequence alignment of AtRAP homologues in higher plants. By Blast search, AtRAP homologues in Arabidopsis (*Arabidopsis thaliana*, At2g31890, GI: 817747), Moss (*Physcomitrella patens*, GI: 168040935), Grape (*Vitis vinifera*, GI: 225434251), Poplar (*Populus trichocarpa*, GI: 224117838), Rice (*Oryza sativa Japonica*, GI: 115453599), Brachypodium (*Brachypodium distachyon*, GI: 357161383), Glycine (*Glycine max*, GI: 356506291) and Ricinus (*Ricinus communis*, GI: 255585295) were obtained from NCBI (www.ncbi.nlm.nih.gov). The multiple sequence alignment was performed by using ClustalW (Thompson et al., 2002) and illustrated by Genedoc (http://www.psc.edu/biomed/genedoc). Black boxes indicate strictly conserved amino acids, and gray boxes closely related ones. The conserved RAP domain/ Restriction_endonuclease_like region is labeled by a black box.

Finally, the AtRAP protein becomes interesting in a completely different context. The *A. thaliana AtRAP* mRNA was found to be a specific target of *Pseudomonas syringae* – mediated induction of AtlsiRNA-1, which is one of the novel classes of endogenous siRNA (Katiyar-Agarwal et al., 2007). This by bacterial infection or specific growth conditions induced AtlsiRNA employs a unique mechanism to degrade *AtRAP* mRNA. Accordingly, AtRAP was speculated to act as a negative regulator which peformes a role in disease resistance, which is supported by enhanced resistance to *Pseudomonas syringae* infections in the *AtRAP-1* mutant (Katiyar-Agarwal et al., 2007).

Considering the here identified function of AtRAP in chloroplast16*S* rRNA maturation and therefore in chloroplast translation one might assume that *Pseudomonas*-infected plant cells protect themselves by down regulating chloroplast translation via a siRNA-based mechanism. This would subsequently reduce the supply of bacteria with nutrients and limit their further spread. Therefore, it would be interesting to investigate if the level of mature 16*S* rRNA in *A. thaliana* wild-types upon *Pseudomonas* infection are similarily to *AtRAP* reduced.

Taken together, data obtained for AtRAP in course of this study likely provide the starting point of further important investigations on its role in pathogen defence and the function of its homologues in other Streptophytes.

5 REFERENCES

Adamska, I., Kruse, E., and Kloppstech, K. (2001). Stable insertion of the early light-induced proteins into etioplast membranes requires chlorophyll a. *J Biol Chem* **276**, 8582-7.

Allen, J. F., de Paula, W. B., Puthiyaveetil, S., and Nield, J. (2011). A structural phylogenetic map for chloroplast photosynthesis. *Trends Plant Sci* **16**, 645-55.

Anbudurai, P. R., and Pakrasi, H. B. (1993). Mutational analysis of the PsbL protein of photosystem II in the cyanobacterium *Synechocystis* sp. PCC 6803. *Z Naturforsch C* **48**, 267-74.

Andersson, U., Heddad, M., and Adamska, I. (2003). Light stress-induced one-helix protein of the chlorophyll a/b-binding family associated with photosystem I. *Plant Physiol* **132**, 811-20.

Armbrust, E. V., Berges, J. A., Bowler, C., Green, B. R., Martinez, D., Putnam, N. H., Zhou, S., Allen, A. E., Apt, K. E., Bechner, M., Brzezinski, M. A., Chaal, B. K., Chiovitti, A., Davis, A. K., Demarest, M. S., Detter, J. C., Glavina, T., Goodstein, D., Hadi, M. Z., Hellsten, U., Hildebrand, M., Jenkins, B. D., Jurka, J., Kapitonov, V. V., Kroger, N., Lau, W. W., Lane, T. W., Larimer, F. W., Lippmeier, J. C., Lucas, S., Medina, M., Montsant, A., Obornik, M., Parker, M. S., Palenik, B., Pazour, G. J., Richardson, P. M., Rynearson, T. A., Saito, M. A., Schwartz, D. C., Thamatrakoln, K., Valentin, K., Vardi, A., Wilkerson, F. P., and Rokhsar, D. S. (2004). The genome of the diatom *Thalassiosira pseudonana*: ecology, evolution, and metabolism. *Science* **306**, 79-86.

Arraiano, C. M., Andrade, J. M., Domingues, S., Guinote, I. B., Malecki, M., Matos, R. G., Moreira, R. N., Pobre, V., Reis, F. P., Saramago, M., Silva, I. J., and Viegas, S. C. (2010). The critical role of RNA processing and degradation in the control of gene expression. *FEMS Microbiol Rev* **34**, 883-923.

Auchincloss, A. H., Zerges, W., Perron, K., Girard-Bascou, J., and Rochaix, J. D. (2002). Characterization of Tbc2, a nucleus-encoded factor specifically required for translation of the chloroplast psbC mRNA in *Chlamydomonas reinhardtii*. *J Cell Biol* **157**, 953-62.

Balczun, C., Bunse, A., Hahn, D., Bennoun, P., Nickelsen, J., and Kück, U. (2005). Two adjacent nuclear genes are required for functional complementation of a chloroplast *trans*-splicing mutant from *Chlamydomonas reinhardtii*. *Plant J* **43**, 636-48.

Balczun, C., Bunse, A., Schwarz, C., Piotrowski, M., and Kück, U. (2006). Chloroplast heat shock protein Cpn60 from *Chlamydomonas reinhardtii* exhibits a novel function as a group II intron-specific RNA-binding protein. *FEBS Lett* **580**, 4527-32.

Barkan, A. (1989). Tissue-dependent plastid RNA splicing in maize: transcripts from four plastid genes are predominantly unspliced in leaf meristems and roots. *Plant Cell* **1**, 437-45.

Barkan, A. (1993). Nuclear mutants of maize with defects in chloroplast polysome assembly have altered chloroplast RNA metabolism. *Plant Cell* **5**, 389-402.

Barkan, A., and Goldschmidt-Clermont, M. (2000). Participation of nuclear genes in chloroplast gene expression. *Biochimie* **82**, 559-72.

Barkan, A., Walker, M., Nolasco, M., and Johnson, D. (1994). A nuclear mutation in maize blocks the processing and translation of several chloroplast mRNAs and provides evidence for the differential translation of alternative mRNA forms. *EMBO J* **13**, 3170-81.

Beick, S., Schmitz-Linneweber, C., Williams-Carrier, R., Jensen, B., and Barkan, A. (2008). The pentatricopeptide repeat protein PPR5 stabilizes a specific tRNA precursor in maize chloroplasts. *Mol Cell Biol* **28**, 5337-47.

Beligni, M. V., and Mayfield, S. P. (2008). *Arabidopsis thaliana* mutants reveal a role for CSP41a and CSP41b, two ribosome-associated endonucleases, in chloroplast ribosomal RNA metabolism. *Plant Mol Biol* **67**, 389-401.

Bellaoui, M., Keddie, J. S., and Gruissem, W. (2003). DCL is a plant-specific protein required for plastid ribosomal RNA processing and embryo development. *Plant Mol Biol* **53**, 531-43.

Bennoun, P., Masson, A., and Delosme, M. (1980). A method for complementation analysis of nuclear and chloroplast mutants of photosynthesis in *chlamydomonas*. *Genetics* **95**, 39-47.

Bentley, F. K., Luo, H., Dilbeck, P., Burnap, R. L., and Eaton-Rye, J. J. (2008). Effects of inactivating psbM and psbT on photodamage and assembly of photosystem II in *Synechocystis* sp. PCC 6803. *Biochemistry* **47**, 11637-46.

Bisanz, C., Begot, L., Carol, P., Perez, P., Bligny, M., Pesey, H., Gallois, J. L., Lerbs-Mache, S., and Mache, R. (2003). The Arabidopsis nuclear *DAL* gene encodes a chloroplast protein which is required for the maturation of the plastid ribosomal RNAs and is essential for chloroplast differentiation. *Plant Mol Biol* **51**, 651-63.

Blatch, G. L., and Lassle, M. (1999). The tetratricopeptide repeat: a structural motif mediating protein-protein interactions. *Bioessays* **21**, 932-9.

Boekema, E. J., van Breemen, J. F., van Roon, H., and Dekker, J. P. (2000). Conformational changes in photosystem II supercomplexes upon removal of extrinsic subunits. *Biochemistry* **39**, 12907-15.

Bohne, A. V., Irihimovitch, V., Weihe, A., and Stern, D. B. (2006). *Chlamydomonas reinhardtii* encodes a single sigma70-like factor which likely functions in chloroplast transcription. *Curr Genet* **49**, 333-40.

Bollenbach, T. J., Lange, H., Gutierrez, R., Erhardt, M., Stern, D. B., and Gagliardi, D. (2005). RNR1, a 3'-5' exoribonuclease belonging to the RNR superfamily, catalyzes 3' maturation of chloroplast ribosomal RNAs in *Arabidopsis thaliana*. *Nucleic Acids Res* **33**, 2751-63.

Bonardi, V., Pesaresi, P., Becker, T., Schleiff, E., Wagner, R., Pfannschmidt, T., Jahns, P., and Leister, D. (2005). Photosystem II core phosphorylation and photosynthetic acclimation require two different protein kinases. *Nature* **437**, 1179-82.

Boudreau, E., Nickelsen, J., Lemaire, S. D., Ossenbühl, F., and Rochaix, J. D. (2000). The *Nac2* gene of Chlamydomonas encodes a chloroplast TPR-like protein involved in *psbD* mRNA stability. *EMBO J* **19**, 3366-76.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248-54.

Bruick, R. K., and Mayfield, S. P. (1998). Processing of the *psbA* 5' untranslated region in *Chlamydomonas reinhardtii* depends upon factors mediating ribosome association. *J Cell Biol* **143**, 1145-53.

Cahoon, B. A., and Stern, D. B. (2001). Plastid transcription: a menage a trois? *Trends Plant Sci* **6**, 45-6.

Cai, W., Ma, J., Chi, W., Zou, M., Guo, J., Lu, C., and Zhang, L. (2010). Cooperation of LPA3 and LPA2 is essential for photosystem II assembly in Arabidopsis. *Plant Physiol* **154**, 109-20.

Carter, M. L., Smith, A. C., Kobayashi, H., Purton, S., and Herrin, D. L. (2004). Structure, circadian regulation and bioinformatic analysis of the unique sigma factor gene in *Chlamydomonas reinhardtii*. *Photosynth Res* **82**, 339-49.

Cheng, Z. F., and Deutscher, M. P. (2005). An important role for RNase R in mRNA decay. *Mol Cell* **17**, 313-8.

Choquet, Y., and Vallon, O. (2000). Synthesis, assembly and degradation of thylakoid membrane proteins. *Biochimie* **82**, 615-34.

Choquet, Y., Wostrikoff, K., Rimbault, B., Zito, F., Girard-Bascou, J., Drapier, D., and Wollman, F. A. (2001). Assembly-controlled regulation of chloroplast gene translation. *Biochem Soc Trans* **29**, 421-6.

D'Andrea, L. D., and Regan, L. (2003). TPR proteins: the versatile helix. *Trends Biochem Sci* **28**, 655-62.

Das, A. K., Cohen, P. W., and Barford, D. (1998). The structure of the tetratricopeptide repeats of protein phosphatase 5: implications for TPR-mediated protein-protein interactions. *EMBO J* **17**, 1192-9.

Dauvillee, D., Stampacchia, O., Girard-Bascou, J., and Rochaix, J. D. (2003). Tab2 is a novel conserved RNA binding protein required for translation of the chloroplast *psaB* mRNA. *EMBO J* **22**, 6378-88.

Davies, B. W., Kohrer, C., Jacob, A. I., Simmons, L. A., Zhu, J., Aleman, L. M., Rajbhandary, U. L., and Walker, G. C. (2010). Role of *Escherichia coli* YbeY, a highly conserved protein, in rRNA processing. *Mol Microbiol* **78**, 506-18.

de Longevialle, A. F., Hendrickson, L., Taylor, N. L., Delannoy, E., Lurin, C., Badger, M., Millar, A. H., and Small, I. (2008). The pentatricopeptide repeat gene *OTP51* with two LAGLIDADG motifs is required for the *cis*-splicing of plastid *ycf3* intron 2 in *Arabidopsis thaliana*. *Plant J* **56**, 157-68.

Dekker, J. P., and Boekema, E. J. (2005). Supramolecular organization of thylakoid membrane proteins in green plants. *Biochim Biophys Acta* **1706**, 12-39.

Derelle, E., Ferraz, C., Rombauts, S., Rouze, P., Worden, A. Z., Robbens, S., Partensky, F., Degroeve, S., Echeynie, S., Cooke, R., Saeys, Y., Wuyts, J., Jabbari, K., Bowler, C., Panaud, O., Piegu, B., Ball, S. G., Ral, J. P., Bouget, F. Y., Piganeau, G., De Baets, B., Picard, A., Delseny, M., Demaille, J., Van de Peer, Y., and Moreau, H. (2006). Genome analysis of the smallest free-living eukaryote *Ostreococcus tauri* unveils many unique features. *Proc Natl Acad Sci U S A* **103**, 11647-52.

Deshpande, N. N., Bao, Y., and Herrin, D. L. (1997). Evidence for light/redox-regulated splicing of *psbA* pre-RNAs in Chlamydomonas chloroplasts. *RNA* **3**, 37-48.

Drapier, D., Girard-Bascou, J., and Wollman, F. A. (1992). Evidence for nuclear control of the expression of the *atp*A and *atp*B chloroplast genes in *Chlamydomonas*. *Plant Cell* **4**, 283-295.

Drapier, D., Suzuki, H., Levy, H., Rimbault, B., Kindle, K. L., Stern, D. B., and Wollman, F. A. (1998). The chloroplast *atp*A gene cluster in *Chlamydomonas reinhardtii*. Functional analysis of a polycistronic transcription unit. *Plant Physiol* **117**, 629-41.

Eberhard, S., Loiselay, C., Drapier, D., Bujaldon, S., Girard-Bascou, J., Kuras, R., Choquet, Y., and Wollman, F. A. (2011). Dual functions of the nucleus-encoded factor TDA1 in trapping and translation activation of *atpA* transcripts in *Chlamydomonas reinhardtii* chloroplasts. *Plant J* **67**, 1055-66.

Emanuel, C., Weihe, A., Graner, A., Hess, W. R., and Börner, T. (2004). Chloroplast development affects expression of phage-type RNA polymerases in barley leaves. *Plant J* **38**, 460-72.

Emanuelsson, O., Nielsen, H., Brunak, S., and von Heijne, G. (2000). Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J Mol Biol* **300**, 1005-16.

Emanuelsson, O., Nielsen, H., and von Heijne, G. (1999). ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. *Protein Sci* **8**, 978-84.

Enami, I., Okumura, A., Nagao, R., Suzuki, T., Iwai, M., and Shen, J. R. (2008). Structures and functions of the extrinsic proteins of photosystem II from different species. *Photosynth Res* **98**, 349-63.

Felder, S., Meierhoff, K., Sane, A. P., Meurer, J., Driemel, C., Plucken, H., Klaff, P., Stein, B., Bechtold, N., and Westhoff, P. (2001). The nucleus-encoded *HCF107* gene of Arabidopsis provides a link between intercistronic RNA processing and the accumulation of translation-competent *psbH* transcripts in chloroplasts. *Plant Cell* **13**, 2127-41.

Ferreira, K. N., Iverson, T. M., Maghlaoui, K., Barber, J., and Iwata, S. (2004). Architecture of the photosynthetic oxygen-evolving center. *Science* **303**, 1831-8.

Fischer, N., Stampacchia, O., Redding, K., and Rochaix, J. D. (1996). Selectable marker recycling in the chloroplast. *Mol Gen Genet* **251**, 373-80.

Fisk, D. G., Walker, M. B., and Barkan, A. (1999). Molecular cloning of the maize gene *crp1* reveals similarity between regulators of mitochondrial and chloroplast gene expression. *EMBO J* **18**, 2621-30.

Gamble, P. E., and Mullet, J. E. (1989). Blue light regulates the accumulation of two *psbD-psbC* transcripts in barley chloroplasts. *EMBO J* **8**, 2785-94.

García-Cerdán, J. G., Kovács, L., Tóth, T., Kereïche, S., Aseeva, E., Boekema, E. J., Mamedov, F., Funk, C., and Schröder, W. P. (2011). The PsbW protein stabilizes the supramolecular organization of photosystem II in higher plants. *Plant J* **65**, 368-81.

Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R. D., and Bairoch, A. (2003). ExPASy: The proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Res* **31**, 3784-8.

Glanz, S., Bunse, A., Wimbert, A., Balczun, C., and Kück, U. (2006). A nucleosome assembly protein-like polypeptide binds to chloroplast group II intron RNA in *Chlamydomonas reinhardtii*. *Nucleic Acids Res* **34**, 5337-51.

Glanz, S., Jacobs, J., Kock, V., Mishra, A., and Kück, U. (2012). Raa4 is a trans-splicing factor that specifically binds chloroplast *tscA* intron RNA. *Plant J* **69**, 421-31.

Göhrea, V., Ossenbühlb, F., Crèvecoeura, M., Eichackerc, L. A., and Rochaix, J.-D. (2006). One of two alb3 proteins is essential for the assembly of the photosystems and for cell survival in Chlamydomonas. *Plant Cell* **18**, 1454-66.

Goldschmidt-Clermont, M., Girard-Bascou, J., Choquet, Y., and Rochaix, J. D. (1990). *Trans*-splicing mutants of *Chlamydomonas reinhardtii*. *Mol Gen Genet* **223**, 417-25.

Granvogl, B., Reisinger, V., and Eichacker, L. A. (2006). Mapping the proteome of thylakoid membranes by *de novo* sequencing of intermembrane peptide domains. *Proteomics* **6**, 3681-95.

Green, B., and Gantt, E. (2005). Distal and extrinsic photosystem II antennas In: Wydrzynski T, Satoh K. eds. Photosystem II: the light-driven water:plastoquinone oxidoreductase. *Springer: The Netherlands*, 23-44.

Grossman, A. R., Harris, E. E., Hauser, C., Lefebvre, P. A., Martinez, D., Rokhsar, D., Shrager, J., Silflow, C. D., Stern, D., Vallon, O., and Zhang, Z. (2003). *Chlamydomonas reinhardtii* at the crossroads of genomics. *Eukaryot Cell* **2**, 1137-50.

Harris, E. H. (2001). *Chlamydomonas* as a model organism. *Annu Rev Plant Physiol Plant Mol Biol* **52**, 363-406.

Harris, E. H. (2009). "The Chlamydomonas source book," San Diego: Academic Press.

Harris, E. H., Boynton, J. E., and Gillham, N. W. (1994). Chloroplast ribosomes and protein synthesis. *Microbiol Rev* **58**, 700-54.

Harris, E. H., Burkhart, B. D., Gillham, N. W., and Boynton, J. E. (1989). Antibiotic resistance mutations in the chloroplast 16*S* and 23*S* rRNA genes of *Chlamydomonas reinhardtii*: correlation of genetic and physical maps of the chloroplast genome. *Genetics* **123**, 281-92.

Hashimoto, M., Endo, T., Peltier, G., Tasaka, M., and Shikanai, T. (2003). A nucleus-encoded factor, CRR2, is essential for the expression of chloroplast *ndhB* in *Arabidopsis*. *Plant J* **36**, 541-9.

He, Q., and Vermaas, W. (1998). Chlorophyll a availability affects psbA translation and D1 precursor processing in vivo in Synechocystis sp. PCC 6803. *Proc Natl Acad Sci U S A* **95**, 5830-5.

Heddad, M., Noren, H., Reiser, V., Dunaeva, M., Andersson, B., and Adamska, I. (2006). Differential expression and localization of early light-induced proteins in *Arabidopsis*. *Plant Physiol* **142**, 75-87.

Herrin, D. L., and Nickelsen, J. (2004). Chloroplast RNA processing and stability. *Photosynth Res* **82**, 301-14.

Herrmann, J. M., and Neupert, W. (2003). Protein insertion into the inner membrane of mitochondria. *IUBMB Life* **55**, 219-25.

Hirano, T., Kinoshita, N., Morikawa, K., and Yanagida, M. (1990). Snap helix with knob and hole: essential repeats in *S. pombe* nuclear protein nuc2+. *Cell* **60**, 319-28.

Hirose, T., and Sugiura, M. (1996). *Cis*-acting elements and *trans*-acting factors for accurate translation of chloroplast *psbA* mRNAs: development of an *in vitro* translation system from tobacco chloroplasts. *EMBO J* **15**, 1687-95.

Holloway, S. P., and Herrin, D. L. (1998). Processing of a composite large subunit rRNA. Studies with chlamydomonas mutants deficient in maturation of the 23s-like rrna. *Plant Cell* **10**, 1193-206.

Houille-Vernes, L., Rappaport, F., Wollman, F. A., Alric, J., and Johnson, X. (2011). Plastid terminal oxidase 2 (PTOX2) is the major oxidase involved in chlororespiration in Chlamydomonas. *Proc Natl Acad Sci U S A* **108**, 20820-5.

Hutin, C., Nussaume, L., Moise, N., Moya, I., Kloppstech, K., and Havaux, M. (2003). Early light-induced proteins protect Arabidopsis from photooxidative stress. *Proc Natl Acad Sci U S A* **100**, 4921-6.

Ikeuchi, M., Nyhus, K. J., Inoue, Y., and Pakrasi, H. B. (1991). Identities of four low-molecular-mass subunits of the photosystem I complex from *Anabaena variabilis* ATCC 29413. Evidence for the presence of the *psaI* gene product in a cyanobacterial complex. *FEBS Lett* **287**, 5-9.

Inoue-Kashino, N., Takahashi, T., Ban, A., Sugiura, M., Takahashi, Y., Satoh, K., and Kashino, Y. (2008). Evidence for a stable association of Psb30 (Ycf12) with photosystem II core complex in the cyanobacterium *Synechocystis* sp. PCC 6803. *Photosynth Res* **98**, 323-35.

Isono, K., Niwa, Y., Satoh, K., and Kobayashi, H. (1997). Evidence for transcriptional regulation of plastid photosynthesis genes in *Arabidopsis thaliana* roots. *Plant Physiol* **114**, 623-30.

Iwai, M., Katayama, M., and Ikeuchi, M. (2006). Absence of the *psbH* gene product destabilizes the Photosystem II complex and prevents association of the Photosystem II-X protein in the thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1. *Photosynth Res* **87**, 313-22.

Iwai, M., Katoh, H., Katayama, M., and Ikeuchi, M. (2004). PSII-Tc protein plays an important role in dimerization of photosystem II. *Plant Cell Physiol* **45**, 1809-16.

Iwai, M., Suzuki, T., Kamiyama, A., Sakurai, I., Dohmae, N., Inoue, Y., and Ikeuchi, M. (2010). The PsbK subunit is required for the stable assembly and stability of other small subunits in the PSII complex in the thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1. *Plant Cell Physiol* **51**, 554-60.

Jarvis, P., and Soll, J. (2001). Toc, Tic, and chloroplast protein import. *Biochim Biophys Acta* **1541**, 64-79.

Jin, E., Yokthongwattana, K., Polle, J. E., and Melis, A. (2003). Role of the reversible xanthophyll cycle in the photosystem II damage and repair cycle in *Dunaliella salina*. *Plant Physiol* **132**, 352-64.

Jin, E. S., Polle, J. E., and Melis, A. (2001). Involvement of zeaxanthin and of the Cbr protein in the repair of photosystem II from photoinhibition in the green alga *Dunaliella salina*. *Biochim Biophys Acta* **1506**, 244-59.

Johnson, X., Kuras, R., Wollman, F.A., and Vallon, O. (2007). Gene hunting by complementation of pooled Chlamydomonas mutants. . *In14th International Congress of Photosynthesis, J. Allen, ed (Glasgow, UK: Springer)*, 1093-1097.

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**, 234-48.

Kashino, Y., Koike, H., Yoshio, M., Egashira, H., Ikeuchi, M., Pakrasi, H. B., and Satoh, K. (2002). Low-molecular-mass polypeptide components of a photosystem II preparation from the thermophilic cyanobacterium *Thermosynechococcus vulcanus*. *Plant Cell Physiol* **43**, 1366-73.

Katiyar-Agarwal, S., Gao, S., Vivian-Smith, A., and Jin, H. (2007). A novel class of bacteria-induced small RNAs in *Arabidopsis*. *Genes Dev* **21**, 3123-34.

Kato, Y., and Sakamoto, W. (2009). Protein quality control in chloroplasts: a current model of D1 protein degradation in the photosystem II repair cycle. *J Biochem* **146**, 463-9.

Kawakami, K., Umena, Y., Iwai, M., Kawabata, Y., Ikeuchi, M., Kamiya, N., and Shen, J. R. (2011a). Roles of PsbI and PsbM in photosystem II dimer formation and stability studied by deletion mutagenesis and X-ray crystallography. *Biochim Biophys Acta* **1807**, 319-25.

Kawakami, K., Umena, Y., Kamiya, N., and Shen, J. R. (2011b). Structure of the catalytic, inorganic core of oxygen-evolving photosystem II at 1.9 Å resolution. *J Photochem Photobiol B* **104**, 9-18.

Kim, J., Eichacker, L. A., Rudiger, W., and Mullet, J. E. (1994). Chlorophyll regulates accumulation of the plastid-encoded chlorophyll proteins P700 and D1 by increasing apoprotein stability. *Plant Physiol* **104**, 907-16.

Kim, J., and Mayfield, S. P. (1997). Protein disulfide isomerase as a regulator of chloroplast translational activation. *Science* **278**, 1954-7.

Kishine, M., Takabayashi, A., Munekage, Y., Shikanai, T., Endo, T., and Sato, F. (2004). Ribosomal RNA processing and an RNase R family member in chloroplasts of Arabidopsis. *Plant Mol Biol* **55**, 595-606.

Klinkert, B., Elles, I., and Nickelsen, J. (2006). Translation of chloroplast *psbD* mRNA in Chlamydomonas is controlled by a secondary RNA structure blocking the AUG start codon. *Nucleic Acids Res* **34**, 386-94.

Klinkert, B., Ossenbühl, F., Sikorski, M., Berry, S., Eichacker, L., and Nickelsen, J. (2004). PratA, a periplasmic tetratricopeptide repeat protein involved in biogenesis of photosystem II in *Synechocystis* sp. PCC 6803. *J Biol Chem* **279**, 44639-44.

Komatsu, T., Kawaide, H., Saito, C., Yamagami, A., Shimada, S., Nakazawa, M., Matsui, M., Nakano, A., Tsujimoto, M., Natsume, M., Abe, H., Asami, T., and Nakano, T. (2010). The chloroplast protein BPG2 functions in brassinosteroid-mediated post-transcriptional accumulation of chloroplast rRNA. *Plant J* **61**, 409-22.

Komenda, J., Nickelsen, J., Tichý, M., Prášil, O., Eichacker, L. A., and Nixon, P. J. (2008). The cyanobacterial homologue of HCF136/YCF48 is a component of an early photosystem II assembly complex and is important for both the efficient assembly and repair of photosystem II in *Synechocystis* sp. PCC 6803. *J Biol Chem* **283**, 22390-9.

Kotera, E., Tasaka, M., and Shikanai, T. (2005). A pentatricopeptide repeat protein is essential for RNA editing in chloroplasts. *Nature* **433**, 326-30.

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

Koussevitzky, S., Nott, A., Mockler, T. C., Hong, F., Sachetto-Martins, G., Surpin, M., Lim, J., Mittler, R., and Chory, J. (2007). Signals from chloroplasts converge to regulate nuclear gene expression. *Science* **316**, 715-9.

Kroeger, T. S., Watkins, K. P., Friso, G., van Wijk, K. J., and Barkan, A. (2009). A plant-specific RNA-binding domain revealed through analysis of chloroplast group II intron splicing. *Proc Natl Acad Sci U S A* **106**, 4537-42.

Künstner, P., Guardiola, A., Takahashi, Y., and Rochaix, J. D. (1995). A mutant strain of *Chlamydomonas reinhardtii* lacking the chloroplast photosystem II *psbI* gene grows photoautotrophically. *J Biol Chem* **270**, 9651-4.

Kutschera, U., and Niklas, K. J. (2005). Endosymbiosis, cell evolution, and speciation. *Theory Biosci* **124**, 1-24.

Laemmli, U. K., Beguin, F., and Gujer-Kellenberger, G. (1970). A factor preventing the major head protein of bacteriophage T4 from random aggregation. *J Mol Biol* **47**, 69-85.

Leal-Klevezas, D. S., Martinez-Soriano, J. P., and Nazar, R. N. (2000). Cotranscription of 5S rRNA-tRNA(Arg)(ACG) from Brassica napus chloroplasts and processing of their intergenic spacer. *Gene* **253**, 303-11.

Lee, I., and Hong, W. (2004). RAP--a putative RNA-binding domain. *Trends Biochem Sci* **29**, 567-70.

Lefebvre, P. A., and Silflow, C. D. (1999). *Chlamydomonas*: the cell and its genomes. *Genetics* **151**, 9-14.

Lennartz, K., Plücken, H., Seidler, A., Westhoff, P., Bechtold, N., and Meierhoff, K. (2001). *HCF164* encodes a thioredoxin-like protein involved in the biogenesis of the cytochrome b_6f complex in Arabidopsis. *Plant Cell* **13**, 2539-51.

Lerbs-Mache, S. (2000). Regulation of rDNA transcription in plastids of higher plants. *Biochimie* **82**, 525-35.

Letunic, I., Goodstadt, L., Dickens, N. J., Doerks, T., Schultz, J., Mott, R., Ciccarelli, F., Copley, R. R., Ponting, C. P., and Bork, P. (2002). Recent improvements to the SMART domain-based sequence annotation resource. *Nucleic Acids Res* **30**, 242-4.

Liere, K., Weihe, A., and Börner, T. (2011). The transcription machineries of plant mitochondria and chloroplasts: Composition, function, and regulation. *J Plant Physiol* **168**, 1345-60.

Link, T., Chen, X. Y., Niu, L. H., and Schramm, V. L. (1996). A hypothesis to explain the substrate reactivity of ribosomal and stem-loop RNA with ricin A-chain. *Toxicon* **34**, 1317-24.

Little, M. C., and Hallick, R. B. (1988). Chloroplast *rpoA*, *rpoB*, and *rpoC* genes specify at least three components of a chloroplast DNA-dependent RNA polymerase active in tRNA and mRNA transcription. *J Biol Chem* **263**, 14302-7.

Loiselay, C., Gumpel, N. J., Girard-Bascou, J., Watson, A. T., Purton, S., Wollman, F. A., and Choquet, Y. (2008). Molecular identification and function of *cis*- and *trans*-acting determinants for *petA* transcript stability in *Chlamydomonas reinhardtii* chloroplasts. *Mol Cell Biol* **28**, 5529-42.

Luo, H., and Eaton-Rye, J. J. (2008). Directed mutagenesis of the transmembrane domain of the PsbL subunit of photosystem II in *Synechocystis* sp. PCC 6803. *Photosynth Res* **98**, 337-47.

Majeran, W., Olive, J., Drapier, D., Vallon, O., and Wollman, F. A. (2001). The light sensitivity of ATP synthase mutants of *Chlamydomonas reinhardtii*. *Plant Physiol* **126**, 421-33.

Manuell, A., Beligni, M. V., Yamaguchi, K., and Mayfield, S. P. (2004). Regulation of chloroplast translation: interactions of RNA elements, RNA-binding proteins and the plastid ribosome. *Biochem Soc Trans* **32**, 601-5.

Martin, W., Rujan, T., Richly, E., Hansen, A., Cornelsen, S., Lins, T., Leister, D., Stoebe, B., Hasegawa, M., and Penny, D. (2002). Evolutionary analysis of Arabidopsis, cyanobacterial, and chloroplast genomes reveals plastid phylogeny and thousands of cyanobacterial genes in the nucleus. *Proc Natl Acad Sci U S A* **99**, 12246-51.

Mayfield, K. P., Horvath, R., Lai, J., and Porreca, F. (1995). Increased expression of opioid delta receptors by deoxy conformation heme proteins in NG108-15 cells. *Brain Res* **676**, 358-62.

McCarty, R. E., Evron, Y., and Johnson, E. A. (2000). Te chloroplast ATP synthase: A rotary enzyme? *Annu Rev Plant Physiol Plant Mol Biol* **51**, 83-109.

Meierhoff, K., Felder, S., Nakamura, T., Bechtold, N., and Schuster, G. (2003). HCF152, an Arabidopsis RNA binding pentatricopeptide repeat protein involved in the processing of chloroplast *psbB-psbT-psbH-petB-petD* RNAs. *Plant Cell* **15**, 1480-95.

Merendino, L., Perron, K., Rahire, M., Howald, I., Rochaix, J. D., and Goldschmidt-Clermont, M. (2006). A novel multifunctional factor involved in *trans*-splicing of chloroplast introns in Chlamydomonas. *Nucleic Acids Res* **34**, 262-74.

Meslet-Cladiere, L., and Vallon, O. (2011). Novel shuttle markers for nuclear transformation of the green alga *Chlamydomonas reinhardtii*. *Eukaryot Cell* **10**, 1670-8.

Meurer, J., Plücken, H., Kowallik, K. V., and Westhoff, P. (1998). A nuclear-encoded protein of prokaryotic origin is essential for the stability of photosystem II in *Arabidopsis thaliana*. *EMBO J* **17**, 5286-97.

Minai, L., Wostrikoff, K., Wollman, F. A., and Choquet, Y. (2006). Chloroplast biogenesis of photosystem II cores involves a series of assembly-controlled steps that regulate translation. *Plant Cell* **18**, 159-75.

Monde, R. A., Greene, J. C., and Stern, D. B. (2000). The sequence and secondary structure of the 3'-UTR affect 3'-end maturation, RNA accumulation, and translation in tobacco chloroplasts. *Plant Mol Biol* **44**, 529-42.

Monod, C., Goldschmidt-Clermont, M., and Rochaix, J. D. (1992). Accumulation of chloroplast *psbB* RNA requires a nuclear factor in *Chlamydomonas reinhardtii*. *Mol Gen Genet* **231**, 449-59.

Montane, M. H., and Kloppstech, K. (2000). The family of light-harvesting-related proteins (LHCs, ELIPs, HLIPs): was the harvesting of light their primary function? *Gene* **258**, 1-8.

Morais, F., Barber, J., and Nixon, P. J. (1998). The chloroplast-encoded alpha subunit of cytochrome b-559 is required for assembly of the photosystem two complex in both the light and the dark in *Chlamydomonas reinhardtii*. *J Biol Chem* **273**, 29315-20.

Mullet, J. E. (1993). Dynamic regulation of chloroplast transcription. *Plant Physiol* **103**, 309-13.

Mullet, J. E., Klein, P. G., and Klein, R. R. (1990). Chlorophyll regulates accumulation of the plastid-encoded chlorophyll apoproteins CP43 and D1 by increasing apoprotein stability. *Proc Natl Acad Sci U S A* **87**, 4038-42.

Mulo, P., Sirpio, S., Suorsa, M., and Aro, E. M. (2008). Auxiliary proteins involved in the assembly and sustenance of photosystem II. *Photosynth Res* **98**, 489-501.

Murakami, S., Kuehnle, K., and Stern, D. B. (2005). A spontaneous tRNA suppressor of a mutation in the *Chlamydomonas reinhardtii* nuclear *MCD1* gene required for stability of the chloroplast *petD* mRNA. *Nucleic Acids Res* **33**, 3372-80.

Navarro, J. A., Vera, A., and Flores, R. (2000). A chloroplastic RNA polymerase resistant to tagetitoxin is involved in replication of avocado sunblotch viroid. *Virology* **268**, 218-25.

Neupert, J., Karcher, D., and Bock, R. (2009). Generation of Chlamydomonas strains that efficiently express nuclear transgenes. *Plant J* **57**, 1140-50.

Nickelsen, J., Fleischmann, M., Boudreau, E., Rahire, M., and Rochaix, J. D. (1999). Identification of *cis*-acting RNA leader elements required for chloroplast *psbD* gene expression in Chlamydomonas. *Plant Cell* **11**, 957-70.

Nickelsen, J., and Kück, U. (2000). The unicellular green alga *Chlamydomonas reinhardtii* as an experimental system to study chloroplast RNA metabolism. *Naturwissenschaften* **87**, 97-107.

Nissen, P., Hansen, J., Ban, N., Moore, P. B., and Steitz, T. A. (2000). The structural basis of ribosome activity in peptide bond synthesis. *Science* **289**, 920-30.

Nixon, P. J., Michoux, F., Yu, J., Boehm, M., and Komenda, J. (2010). Recent advances in understanding the assembly and repair of photosystem II. *Ann Bot* **106**, 1-16.

O'Connor, H. E., Ruffle, S. V., Cain, A. J., Deak, Z., Vass, I., Nugent, J. H., and Purton, S. (1998). The 9-kDa phosphoprotein of photosystem II. Generation and characterisation of Chlamydomonas mutants lacking PSII-H and a site-directed mutant lacking the phosphorylation site. *Biochim Biophys Acta* **1364**, 63-72.

Ohad, I., Dal Bosco, C., Herrmann, R. G., and Meurer, J. (2004). Photosystem II proteins PsbL and PsbJ regulate electron flow to the plastoquinone pool. *Biochemistry* **43**, 2297-308.

Ohnishi, N., Kashino, Y., Satoh, K., Ozawa, S., and Takahashi, Y. (2007). Chloroplast-encoded polypeptide PsbT is involved in the repair of primary electron acceptor Q_A of photosystem II during photoinhibition in *Chlamydomonas reinhardtii*. *J Biol Chem* **282**, 7107-15.

Ohnishi, N., and Takahashi, Y. (2001). PsbT polypeptide is required for efficient repair of photodamaged photosystem II reaction center. *J Biol Chem* **276**, 33798-804.

Ohnishi, N., and Takahashi, Y. (2008). Chloroplast-encoded PsbT is required for efficient biogenesis of photosystem II complex in the green alga *Chlamydomonas reinhardtii*. *Photosynth Res* **98**, 315-22.

Okuda, K., Myouga, F., Motohashi, R., Shinozaki, K., and Shikanai, T. (2007). Conserved domain structure of pentatricopeptide repeat proteins involved in chloroplast RNA editing. *Proc Natl Acad Sci U S A* **104**, 8178-83.

Ossenbühl., Hartmann, K., and Nickelsen, J. (2002). A chloroplast RNA binding protein from stromal thylakoid membranes specifically binds to the 5' untranslated region of the *psbA* mRNA. *Eur J Biochem* **269**, 3912-9.

Ostersetzer, O., Cooke, A. M., Watkins, K. P., and Barkan, A. (2005). CRS1, a chloroplast group II intron splicing factor, promotes intron folding through specific interactions with two intron domains. *Plant Cell* **17**, 241-55.

Pakrasi, H. B., De Ciechi, P., and Whitmarsh, J. (1991). Site directed mutagenesis of the heme axial ligands of cytochrome b559 affects the stability of the photosystem II complex. *EMBO J* **10**, 1619-27.

Park, S., Khamai, P., Garcia-Cerdan, J. G., and Melis, A. (2007). REP27, a tetratricopeptide repeat nuclear-encoded and chloroplast-localized protein, functions in D1/32-kD reaction center protein turnover and photosystem II repair from photodamage. *Plant Physiol* **143**, 1547-60.

Park, Y. J., Cho, H. K., Jung, H. J., Ahn, C. S., Kang, H., and Pai, H. S. (2011). PRBP plays a role in plastid ribosomal RNA maturation and chloroplast biogenesis in *Nicotiana benthamiana*. *Planta* **233**, 1073-85.

Peng, L., Ma, J., Chi, W., Guo, J., Zhu, S., Lu, Q., Lu, C., and Zhang, L. (2006). LOW PSII ACCUMULATION1 is involved in efficient assembly of photosystem II in *Arabidopsis thaliana*. *Plant Cell* **18**, 955-69.

Peng, L., Shimizu, H., and Shikanai, T. (2008). The chloroplast NAD(P)H dehydrogenase complex interacts with photosystem I in *Arabidopsis*. *J Biol Chem* **283**, 34873-9.

Perron, K., Goldschmidt-Clermont, M., and Rochaix, J. D. (1999). A factor related to pseudouridine synthases is required for chloroplast group II intron *trans*-splicing in *Chlamydomonas reinhardtii*. *EMBO J* **18**, 6481-90.

Perron, K., Goldschmidt-Clermont, M., and Rochaix, J. D. (2004). A multiprotein complex involved in chloroplast group II intron splicing. *RNA* **10**, 704-11.

Pesaresi, P., Hertle, A., Pribil, M., Kleine, T., Wagner, R., Strissel, H., Ihnatowicz, A., Bonardi, V., Scharfenberg, M., Schneider, A., Pfannschmidt, T., and Leister, D. (2009). *Arabidopsis* STN7 kinase provides a link between short- and long-term photosynthetic acclimation. *Plant Cell* **21**, 2402-23.

Pesaresi, P., Masiero, S., Eubel, H., Braun, H. P., Bhushan, S., Glaser, E., Salamini, F., and Leister, D. (2006). Nuclear photosynthetic gene expression is synergistically modulated by rates of protein synthesis in chloroplasts and mitochondria. *Plant Cell* **18**, 970-91.

Pfalz, J., Bayraktar, O. A., Prikryl, J., and Barkan, A. (2009). Site-specific binding of a PPR protein defines and stabilizes 5' and 3' mRNA termini in chloroplasts. *EMBO J* **28**, 2042-52.

Pfalz, J., Liere, K., Kandlbinder, A., Dietz, K. J., and Oelmuller, R. (2006). pTAC2, -6, and -12 are components of the transcriptionally active plastid chromosome that are required for plastid gene expression. *Plant Cell* **18**, 176-97.

Pfannschmidt, T., and Link, G. (1997). The A and B forms of plastid DNA-dependent RNA polymerase from mustard (*Sinapis alba* L.) transcribe the same genes in a different developmental context. *Mol Gen Genet* **257**, 35-44.

Pfannschmidt, T., Nilsson, A., Tullberg, A., Link, G., and Allen, J. F. (1999). Direct transcriptional control of the chloroplast genes *psbA* and *psaAB* adjusts photosynthesis to light energy distribution in plants. *IUBMB Life* **48**, 271-6.

Pfannschmidt, T., Ogrzewalla, K., Baginsky, S., Sickmann, A., Meyer, H. E., and Link, G. (2000). The multisubunit chloroplast RNA polymerase A from mustard (Sinapis alba L.). Integration of a prokaryotic core into a larger complex with organelle-specific functions. *Eur J Biochem* **267**, 253-61.

Prapapanich, V., Chen, S., Toran, E. J., Rimerman, R. A., and Smith, D. F. (1996). Mutational analysis of the hsp70-interacting protein Hip. *Mol Cell Biol* **16**, 6200-7.

Price, C. A., Hadjeb, N., Newman, L. A., and Reardon, E. M. (1995). Chromoplasts. *Methods Cell Biol* **50**, 189-207.

Punta, M., Coggill, P. C., Eberhardt, R. Y., Mistry, J., Tate, J., Boursnell, C., Pang, N., Forslund, K., Ceric, G., Clements, J., Heger, A., Holm, L., Sonnhammer, E. L., Eddy, S. R., Bateman, A., and Finn, R. D. (2012). The Pfam protein families database. *Nucleic Acids Res* **40**, D290-301.

Puthiyaveetil, S., Kavanagh, T. A., Cain, P., Sullivan, J. A., Newell, C. A., Gray, J. C., Robinson, C., van der Giezen, M., Rogers, M. B., and Allen, J. F. (2008). The ancestral symbiont sensor kinase CSK links photosynthesis with gene expression in chloroplasts. *Proc Natl Acad Sci U S A* **105**, 10061-6.

Rapp, J. C., Baumgartner, B. J., and Mullet, J. (1992). Quantitative analysis of transcription and RNA levels of 15 barley chloroplast genes. Transcription rates and mRNA levels vary over 300-fold; predicted mRNA stabilities vary 30-fold. *J Biol Chem* **267**, 21404-11.

Rattanachaikunsopon, P., Rosch, C., and Kuchka, M. R. (1999). Cloning and characterization of the nuclear *AC115* gene of *Chlamydomonas reinhardtii*. *Plant Mol Biol* **39**, 1-10.

Raynaud, C., Loiselay, C., Wostrikoff, K., Kuras, R., Girard-Bascou, J., Wollman, F. A., and Choquet, Y. (2007). Evidence for regulatory function of nucleus-encoded factors on mRNA stabilization and translation in the chloroplast. *Proc Natl Acad Sci U S A* **104**, 9093-8.

Rivier, C., Goldschmidt-Clermont, M., and Rochaix, J. D. (2001). Identification of an RNA-protein complex involved in chloroplast group II intron *trans*-splicing in Chlamydomonas reinhardtii. *EMBO J* **20**, 1765-73.

Rochaix, J. D. (1995). *Chlamydomonas reinhardtii* as the photosynthetic yeast. *Annu Rev Genet* **29**, 209-30.

Rochaix, J. D. (2011). Assembly of the photosynthetic apparatus. *Plant Physiol* **155**, 1493-500.

Rolland, N., Dorne, A. J., Amoroso, G., Sultemeyer, D. F., Joyard, J., and Rochaix, J. D. (1997). Disruption of the plastid *ycf10* open reading frame affects uptake of inorganic carbon in the chloroplast of Chlamydomonas. *EMBO J* **16**, 6713-26.

Rott, R., Liveanu, V., Drager, R. G., Stern, D. B., and Schuster, G. (1998). The sequence and structure of the 3'-untranslated regions of chloroplast transcripts are important determinants of mRNA accumulation and stability. *Plant Mol Biol* **36**, 307-14.

Ruwe, H., and Schmitz-Linneweber, C. (2012). Short non-coding RNA fragments accumulating in chloroplasts: footprints of RNA binding proteins? *Nucleic Acids Res* **40**, 3106-16.

Sambrook J, R. D. (2001). "Molecular Cloning: A laboratory manual," Cold Spring Harbor Laboratory Press, New York.

Sane, A. P., Stein, B., and Westhoff, P. (2005). The nuclear gene *HCF107* encodes a membrane-associated R-TPR (RNA tetratricopeptide repeat)-containing protein involved in expression of the plastidial *psbH* gene in Arabidopsis. *Plant J* **42**, 720-30.

Sato, S., Nakamura, Y., Kaneko, T., Asamizu, E., and Tabata, S. (1999). Complete structure of the chloroplast genome of *Arabidopsis thaliana*. *DNA Res* **6**, 283-90.

Schägger, H., and von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* **166**, 368-79.

Schlodder, E., Coleman, W. J., Nixon, P. J., Cohen, R. O., Renger, T., and Diner, B. A. (2008). Site-directed mutations at D1-His198 and D1-Thr179 of photosystem II in *Synechocystis* sp. PCC 6803: deciphering the spectral properties of the PSII reaction centre. *Philos Trans R Soc Lond B Biol Sci* **363**, 1197-202; discussion 1202.

Schmitz-Linneweber, C., and Small, I. (2008). Pentatricopeptide repeat proteins: a socket set for organelle gene expression. *Trends Plant Sci* **13**, 663-70.

Schmitz-Linneweber, C., Williams-Carrier, R., and Barkan, A. (2005). RNA immunoprecipitation and microarray analysis show a chloroplast pentatricopeptide repeat protein to be associated with the 5' region of mRNAs whose translation it activates. *Plant Cell* **17**, 2791-804.

Schmitz-Linneweber, C., Williams-Carrier, R. E., Williams-Voelker, P. M., Kroeger, T. S., Vichas, A., and Barkan, A. (2006). A pentatricopeptide repeat protein facilitates the *trans*-splicing of the maize chloroplast *rps12* pre-mRNA. *Plant Cell* **18**, 2650-63.

Schottkowski, M., Gkalympoudis, S., Tzekova, N., Stelljes, C., Schünemann, D., Ankele, E., and Nickelsen, J. (2009a). Interaction of the periplasmic PratA factor and the PsbA (D1) protein during biogenesis of photosystem II in *Synechocystis* sp. PCC 6803. *J Biol Chem* **284**, 1813-9.

Schottkowski, M., Ratke, J., Oster, U., Nowaczyk, M., and Nickelsen, J. (2009b). Pitt, a novel tetratricopeptide repeat protein involved in light-dependent chlorophyll biosynthesis and thylakoid membrane biogenesis in *Synechocystis* sp. PCC 6803. *Mol Plant* **2**, 1289-97.

Schroda, M., Beck, C. F., and Vallon, O. (2002). Sequence elements within an *HSP70* promoter counteract transcriptional transgene silencing in Chlamydomonas. *Plant J* **31**, 445-55.

Schult, K., Meierhoff, K., Paradies, S., Töller, T., Wolff, P., and Westhoff, P. (2007). The nuclear-encoded factor HCF173 is involved in the initiation of translation of the psbA mRNA in *Arabidopsis thaliana*. *Plant Cell* **19**, 1329-46.

Schwarz, C., Elles, I., Kortmann, J., Piotrowski, M., and Nickelsen, J. (2007). 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. *Plant Cell* **19**, 3627-39.

Schwenkert, S., Umate, P., Dal Bosco, C., Volz, S., Mlcochová, L., Zoryan, M., Eichacker, L. A., Ohad, I., Herrmann, R. G., and Meurer, J. (2006). PsbI affects the stability, function, and phosphorylation patterns of photosystem II assemblies in tobacco. *J Biol Chem* **281**, 34227-38.

Sessions, A., Burke, E., Presting, G., Aux, G., McElver, J., Patton, D., Dietrich, B., Ho, P., Bacwaden, J., Ko, C., Clarke, J. D., Cotton, D., Bullis, D., Snell, J., Miguel, T., Hutchison, D., Kimmerly, B., Mitzel, T., Katagiri, F., Glazebrook, J., Law, M., and Goff, S. A. (2002). A high-throughput Arabidopsis reverse genetics system. *Plant Cell* **14**, 2985-94.

Severinov, K., Mustaev, A., Kukarin, A., Muzzin, O., Bass, I., Darst, S. A., and Goldfarb, A. (1996). Structural modules of the large subunits of RNA polymerase. Introducing archaebacterial and chloroplast split sites in the beta and beta' subunits of *Escherichia coli* RNA polymerase. *J Biol Chem* **271**, 27969-74.

Sheen, J. (1999). C4 Gene Expression. *Annu Rev Plant Physiol Plant Mol Biol* **50**, 187-217.

Shi, L. X., Hall, M., Funk, C., and Schröder, W. P. (2012). Photosystem II, a growing complex: updates on newly discovered components and low molecular mass proteins. *Biochim Biophys Acta* **1817**, 13-25.

Shi, L. X., Lorkovic, Z. J., Oelmuller, R., and Schröder, W. P. (2000). The low molecular mass PsbW protein is involved in the stabilization of the dimeric photosystem II complex in *Arabidopsis thaliana*. *J Biol Chem* **275**, 37945-50.

Shimogawara, K., Fujiwara, S., Grossman, A., and Usuda, H. (1998). High-efficiency transformation of *Chlamydomonas reinhardtii* by electroporation. *Genetics* **148**, 1821-8.

Sikorski, R. S., Boguski, M. S., Goebl, M., and Hieter, P. (1990). A repeating amino acid motif in CDC23 defines a family of proteins and a new relationship among genes required for mitosis and RNA synthesis. *Cell* **60**, 307-17.

Sirpiö, S., Allahverdiyeva, Y., Suorsa, M., Paakkarinen, V., Vainonen, J., Battchikova, N., and Aro, E. M. (2007). TLP18.3, a novel thylakoid lumen protein regulating photosystem II repair cycle. *Biochem J* **406**, 415-25.

Sizova, I. A., Lapina, T. V., Frolova, O. N., Alexandrova, N. N., Akopiants, K. E., and Danilenko, V. N. (1996). Stable nuclear transformation of *Chlamydomonas reinhardtii* with a *Streptomyces rimosus* gene as the selective marker. *Gene* **181**, 13-8.

Small, I., Peeters, N., Legeai, F., and Lurin, C. (2004). Predotar: a tool for rapidly screening proteomes for N-terminal targeting sequences. *Proteomics* **4**, 1581-90.

Small, I. D., and Peeters, N. (2000). The PPR motif - a TPR-related motif prevalent in plant organellar proteins. *Trends Biochem Sci* **25**, 46-7.

Smith, A. C., Purton, S. (2002). The transcriptional apparatus of algal plastids. *Euro. J. Phycol.*, 301-311.

Somanchi, A., Barnes, D., and Mayfield, S. P. (2005). A nuclear gene of *Chlamydomonas reinhardtii*, *Tba1*, encodes a putative oxidoreductase required for translation of the chloroplast *psbA* mRNA. *Plant J* **42**, 341-52.

Stanke, M., Steinkamp, R., Waack, S., and Morgenstern, B. (2004). AUGUSTUS: a web server for gene finding in eukaryotes. *Nucleic Acids Res* **32**, W309-12.

Stanke, M., and Waack, S. (2003). Gene prediction with a hidden Markov model and a new intron submodel. *Bioinformatics* **19 Suppl 2**, ii215-25.

Stern, D. B., Goldschmidt-Clermont, M., and Hanson, M. R. (2010). Chloroplast RNA metabolism. *Annu Rev Plant Biol* **61**, 125-55.

Stoppel, R., Lezhneva, L., Schwenkert, S., Torabi, S., Felder, S., Meierhoff, K., Westhoff, P., and Meurer, J. (2011). Recruitment of a ribosomal release factor for light- and stress-dependent regulation of petB transcript stability in Arabidopsis chloroplasts. *Plant Cell* **23**, 2680-95.

Stoppel, R., and Meurer, J. (2012). The cutting crew - ribonucleases are key players in the control of plastid gene expression. *J Exp Bot* **63**, 1663-73.

Strittmatter, G., and Kössel, H. (1984). Cotranscription and processing of 23*S*, 4.5*S* and 5*S* rRNA in chloroplasts from *Zea mays*. *Nucleic Acids Res* **12**, 7633-47.

Sugiura, M., Boussac, A., Noguchi, T., and Rappaport, F. (2008). Influence of histidine-198 of the D1 subunit on the properties of the primary electron donor, P680, of photosystem II in *Thermosynechococcus elongatus*. *Biochim Biophys Acta* **1777**, 331-42.

Sugiura, M., Harada, S., Manabe, T., Hayashi, H., Kashino, Y., and Boussac, A. (2010). Psb30 contributes to structurally stabilise the Photosystem II complex in the thermophilic cyanobacterium *Thermosynechococcus elongatus*. *Biochim Biophys Acta* **1797**, 1546-54.

Suorsa, M., Regel, R. E., Paakkarinen, V., Battchikova, N., Herrmann, R. G., and Aro, E. M. (2004). Protein assembly of photosystem II and accumulation of subcomplexes in the absence of low molecular mass subunits PsbL and PsbJ. *Eur J Biochem* **271**, 96-107.

Suzuki, J. Y., Ytterberg, A. J., Beardslee, T. A., Allison, L. A., Wijk, K. J., and Maliga, P. (2004). Affinity purification of the tobacco plastid RNA polymerase and *in vitro* reconstitution of the holoenzyme. *Plant J* **40**, 164-72.

Swiatek, M., Regel, R. E., Meurer, J., Wanner, G., Pakrasi, H. B., Ohad, I., and Herrmann, R. G. (2003). Effects of selective inactivation of individual genes for low-molecular-mass subunits on the assembly of photosystem II, as revealed by chloroplast transformation: the *psbEFLJ*operon in *Nicotiana tabacum*. *Mol Genet Genomics* **268**, 699-710.

Takahashi, Y., Matsumoto, H., Goldschmidt-Clermont, M., and Rochaix, J. D. (1994). Directed disruption of the Chlamydomonas chloroplast *psbK* gene destabilizes the photosystem II reaction center complex. *Plant Mol Biol* **24**, 779-88.

Thidholm, E., Lindström, V., Tissier, C., Robinson, C., Schröder, W. P., and Funk, C. (2002). Novel approach reveals localisation and assembly pathway of the PsbS and PsbW proteins into the photosystem II dimer. *FEBS Lett* **513**, 217-22.

Thompson, J. D., Gibson, T. J., and Higgins, D. G. (2002). Multiple sequence alignment using ClustalW and ClustalX. *Curr Protoc Bioinformatics* **Chapter 2**, Unit 2 3.

Timmis, J. N., Ayliffe, M. A., Huang, C. Y., and Martin, W. (2004). Endosymbiotic gene transfer: organelle genomes forge eukaryotic chromosomes. *Nat Rev Genet* **5**, 123-35.

Trempe, M. R., and Glitz, D. G. (1981). Chloroplast ribosome structure. Electron microscopy of ribosomal subunits and localization of N^6,N^6-dimethyladenosine by immunoelectronmicroscopy. *J Biol Chem* **256**, 11873-9.

Umate, P., Schwenkert, S., Karbat, I., Dal Bosco, C., Mlcòchová, L., Volz, S., Zer, H., Herrmann, R. G., Ohad, I., and Meurer, J. (2007). Deletion of PsbM in tobacco alters the Q_B site properties and the electron flow within photosystem II. *J Biol Chem* **282**, 9758-67.

Uniacke, J., and Zerges, W. (2008). Stress induces the assembly of RNA granules in the chloroplast of *Chlamydomonas reinhardtii*. *J Cell Biol* **182**, 641-6.

Uniacke, J., and Zerges, W. (2009). Chloroplast protein targeting involves localized translation in *Chlamydomonas*. *Proc Natl Acad Sci U S A* **106**, 1439-44.

Vaistij, F. E., Boudreau, E., Lemaire, S. D., Goldschmidt-Clermont, M., and Rochaix, J. D. (2000). Characterization of Mbb1, a nucleus-encoded tetratricopeptide-like repeat protein required for expression of the chloroplast *psbB/psbT/psbH* gene cluster in *Chlamydomonas reinhardtii*. *Proc Natl Acad Sci U S A* **97**, 14813-8.

Walter, M., Kilian, J., and Kudla, J. (2002). PNPase activity determines the efficiency of mRNA 3'-end processing, the degradation of tRNA and the extent of polyadenylation in chloroplasts. *EMBO J* **21**, 6905-14.

Wegener, K. M., Bennewitz, S., Oelmuller, R., and Pakrasi, H. B. (2011). The Psb32 protein aids in repairing photodamaged photosystem II in the cyanobacterium *Synechocystis* 6803. *Mol Plant* **4**, 1052-61.

Wilkins, M. R., Gasteiger, E., Bairoch, A., Sanchez, J. C., Williams, K. L., Appel, R. D., and Hochstrasser, D. F. (1999). Protein identification and analysis tools in the ExPASy server. *Methods Mol Biol* **112**, 531-52.

Williams-Carrier, R., Kroeger, T., and Barkan, A. (2008). Sequence-specific binding of a chloroplast pentatricopeptide repeat protein to its native group II intron ligand. *RNA* **14**, 1930-41.

Yamamoto, Y. Y., Puente, P., and Deng, X. W. (2000). An Arabidopsis cotyledon-specific albino locus: a possible role in 16*S* rRNA maturation. *Plant Cell Physiol* **41**, 68-76.

Yamazaki, H., Tasaka, M., and Shikanai, T. (2004). PPR motifs of the nucleus-encoded factor, PGR3, function in the selective and distinct steps of chloroplast gene expression in Arabidopsis. *Plant J* **38**, 152-63.

Yehudai-Resheff, S., Hirsh, M., and Schuster, G. (2001). Polynucleotide phosphorylase functions as both an exonuclease and a poly(A) polymerase in spinach chloroplasts. *Mol Cell Biol* **21**, 5408-16.

Yohn, C. B., Cohen, A., Danon, A., and Mayfield, S. P. (1998). A poly(A) binding protein functions in the chloroplast as a message-specific translation factor. *Proc Natl Acad Sci U S A* **95**, 2238-43.

Yukawa, M., Kuroda, H., and Sugiura, M. (2007). A new *in vitro* translation system for non-radioactive assay from tobacco chloroplasts: effect of pre-mRNA processing on translation *in vitro*. *Plant J* **49**, 367-76.

Zhang, L., Paakkarinen, V., van Wijk, K. J., and Aro, E. M. (1999). Co-translational assembly of the D1 protein into photosystem II. *J Biol Chem* **274**, 16062-7.

Zhang, L., Paakkarinen, V., van Wijk, K. J., and Aro, E. M. (2000). Biogenesis of the chloroplast-encoded D1 protein: regulation of translation elongation, insertion, and assembly into photosystem II. *Plant Cell* **12**, 1769-82.

Zolla, L., Rinalducci, S., and Timperio, A. M. (2007). Proteomic analysis of photosystem I components from different plant species. *Proteomics* **7**, 1866-76.

Zoschke, R., Liere, K., and Börner, T. (2007). From seedling to mature plant: arabidopsis plastidial genome copy number, RNA accumulation and transcription are differentially regulated during leaf development. *Plant J* **50**, 710-22.

6 ANNEX

Sequencing result of inverse PCR product from mutant *45a*, *42b* and *101b*

Vector sequences are indicated by italic letters.

mutant	Sequencing results of PCR products from different digestion	
	***Pst*I**	***Nhe*I**
45a	CTGCAGCCGCCGTCGCCCACCAGGCGTCCGAAGTCG CCCACGTCCCAAATCTCCGTCTCCTCGCCGTGGTTGA CGCACTTG*ctcgctgaggcttgacatgattggtgcgtatgtttgtatgaagctac aggactgatttggcgggctatgagggcgggggaagctctggaagggccgcgatgg ggcgcgcggcgtccagaagggccatacggcccgctggcggcacccatccggtata aaagcccgcgaccccgaacggtgacctccactttcagcgacaaacagcacttatac atacgcgactattctgccgctatacataaccactcagcttagttaanatcccatttttact tgcgaggggggggcg*	AGAACGCGATACCGGGTCAAGGAATCAACATGA ATGCTTGTTCGCATGGCTGCTTGCGTGCGCTGACT GACAACGTTAGTTCCGCTGACTGCCGCAGCCAAA AGGCCCTGCGTCCCGCTGACAAACAAACGCCTTG TGCTCCACTGACTT
42b	GATACCGCCGCTGCAAGCAATTCGGGAGTCAAGGCA TTGGAAAACGGTCATTGGAGGCAGGGCGTGTGTGAC TGTGTGCCAAGGGAACGGAGCGTAAGGAATGATTAG CACAATAGACGCGAGTGTGCGTGTGCCTTGACTGAG CGGCCTGCTTGACGACTGGCGCCTGTGGGCTCCCTGA ATCACGCACATGTACATTTGCACACGGGACCCCATCC CTTGTCTACCCATCCCTTGTGCTCTGACTGCTCTCTAC CACGCATGCACGCGACTTAGACGCGGCCACTCACCC CCACAGCCCGGCCCGGTTGTCGTTCATCACGGCGCCG CCATCCANTATGATGT	GATACCGCCGCTGCAAGCAATTCGGGAGTCAAG GCATTGGAAAACGGTCATTGGAGGCAGGGCGTG TGTGACTGTGTGCCAAGGGAACGGAGCGTAAGG AATGATTAGCACAATAGACGCGAGTGTGCGTGTG CCTTGACTGAGCGGCCTGCTTGACGACTGGCGCC T*gcttaagatcccatcaagcttgcatgccgggcgcgccagaaggagcgcag ccaaaccaggatgatgtttgatggggtatttgagcacttgcaacccttatccgg aagccccctggcccacaaaggctaggcgccaatgcaagcagttcgcangna gcccctggagcggtgccctcctgataaaccggccaggggcctatgttctttac ttttttacaaganaantcnctcaacatcttaaaatggccnggngagtcgacna nnaancccgg*
101a	TTGAGCGGGTGATGCTGCCGTCATAAGGTGACCTGG GTCGCTCCCCGTCTCTTGGCGTTGCCCAGGACGTACC CCGGCCGTTTTCTGTGCTCCACAGGACCCGTCTCCCT CCACTCCCAACCCAAAGGGCGCTTATCCTATGTATGA ACCTCACTCCGCGCTTATAGTCCGCACACGCACACAA ACAAGCACACGTCACGCAGTCCACATGCGCTCGCAC CGTACACGCACACGCAGTCCACACGCACACACACGC ACACGCAGTCCCCACCTGCTCCTGGTACTGCGCCAGC ATGGCCTTCAGGTTCGCCGCCTCGCCCTCCGCCGCCG CCTTCTCCTGCGCGGGTAGAGAACAGGGATGGCATG GACACGCGAAACATCAGCGGTGCAAGTCGGGCTCCG ATATCGTCAGTACAGGCAGTGGTGCGATGCGAACTG TACATACATACGGTATACATCTACAAACCCCCCAAG AGAAGTGCAAATGTTGTTGGCTTTACTGCTATCCACG TTGCAACCTCCCTCCTCTGCCTCGCCTATAACACACG GCTACGTCGCCTGCCCCTCACGCCTACATCGGCCCCC AACCCACCTGCAGGCTCCGGACCTGAGGAGGCGGGC GGGAGCGGGGGGCGAGAGGAGGCGGGAGGCAACAG TCAAGGCCGGTCAGTGCAGTGAGTGGCGAGAAGGCA GGTGTGACTGTACACGGCCAGCATCCGGGAGCAGTG GCGCTCGGGGCGGCAGGGACGG	TTGAGCGGGTGATGCTGCCGTCATAAGGTGACCT GGGTCGCTCCCCGTCTCTTGGCGTTGCCCAGGAC GTACCCCGGCCGTTTTCTGTGCTCCACAGGACCC GTCTCCCTCCACTCCCAACCCAAAGGGCGCTTAT CCTATGTATGAACCTCACTCCGCGCTTATAGTCC GCACACGCACACAAACAAGCACACGTCACGCAG TCCACATGCGCTCGCACCGTACACGCACACGCAG TCCACACGCACACACACGCACACGCAGTCCCCAC CTGCTCCTGGTACTGCGCCAGCATGGCCTTCAGG TTCGCCGCCTCGCCCTCCGCCGCCGCCTTCTCCTG CGCGGGTAGAGAACAGGGATGGCATGGACACGC GAAACATCAGCGGTGCAAGTCGGGCTCCGATATC GTCAGTACAGGCAGTGGTGCGATGCGAACTGTAC ATACATACGGTATACATCTACAAACCCCCCAAGA GAAGTGCAAATGTTGTTGGCTTTACTGCTATCCA CGTTGCAACCTCCC
101b	GAGCGGGTNATGGCGATGTTGGCGCGCTCGCATCAT TGCGTGTAGAGCGGTCGTGCAGCCCGTGGCAAGCCC GCCGAGCCCTG*GCTAGC*GGTACCGCCTGCTTTTAGCT ACAGCTGGCCCAGCAGCGAATCATGCTCCTCAAGTG AAACACCCCTCCTTACACCGGCCCCGCTTCTCGTTAG GTGCTGTGGTCAGTGGCCTACCACAGCCACTCCTGCC CGGAGCTGCTGGACGCAGCCGCGCCCGCCATCGCCT CGCGCCTGGGCCGCTTCTGCCCCTGGGACGCATCCGT	GAGCGGGTNATGGCGATGTTGGCGCGCTCGCATC ATTGCGTGTAGAGCGGTCGTGCAGCCCGTGGCAA GCCCGCCGAGCCCTGGCTAGC*gctagcttaagatcccatcaa gcttgcatgccgggcgcgccagaaggagcgcagccaaaccaggatgatgttt gatggggtatttgagcacttgcaacccttatccggaagccccctggcccacaa aggctaggcgccaatgcaagcagttcgcatgcagccccctggagcggtgccct cctgataaaccggccaggggcctatgttctttacttttttacaagagaagtcac tcaacatcttaaaatggccaggtgagtcgacgagcaagcccggcggatcag*

	GGTGGCGTGGGCCTACGCCAAGCTGGACCACCCGCA CCGCGACCTGTTCGAGTCGCTGCAG*agcgtgcagcaacagcc ggccaacagcaaatcacaagcagctggcttttcatgccgctaattgccgacgcgct taagatcccatcaagcttgcatgccgggcgcgccagaaggagcgcagccaaacca ggatgatgtttgatggggtatttgagcacttgcaacccttatccggaagccccctggcc cacaaaggctaggcgccaatgcaagcagttcgcatgcagcccctggagcggtgcc ctcctgataaaccggccaggggcctatgttctttacttttttacaagagaagtcactca acatcnnnaaatggccaggtgagtcgacgagcaagcccgnnggatcangcagcg ngcttngnantttgacttgcaa*	*gcagcgtgcttgcanatttgaccttgca*

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PUBLICATIONS AND CONFERENCE ABSTRACTS

Publication

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München, den 18. Juni 2012 Fei Wang