Molecular Characterisation of two Putative Photosynthetic Proteins in *Arabidopsis thaliana*

Dissertation

zur Erlangung des Doktorgrades der Fakultät für Biologie

der Ludwig-Maximilians-Universität München

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Juli 2011

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Tag der mündlichen Prüfung: 28. 07. 2011

Summary

Photosynthesis is the biological process, by which photosynthetic organisms convert light energy and carbon dioxide into organic compounds. In plants, photosynthesis takes place in the chloroplast, and this organelle evolved from the ancestor of cyanobacteria by endosymbiosis. The chloroplast proteome contains thousands of proteins, of which most are encoded by the nuclear genome. Transcriptome analysis reveals that nuclear photosynthetic genes and nuclear genes involved in chloroplast gene expression are co-regulated at the transcriptional level. To test this hypothesis, two co-regulated genes with so-far unknown biological function, named as Putative Photosynthetic Protein 1 (PPP1) and 3 (PPP3), were studied by reverse genetic approaches and biochemical analyses in *Arabidopsis thaliana*. The PPP3 knock-out mutants and the PPP3-eGFP overexpressor in *Arabidopsis* have no distinct phenotype. PPP3 can form a small complex attached to thylakoid lamellae, and these interactions might be caused by two protein-protein interaction domains of PPP3 (TRP and PDZ). PPP1, also known as CSP41b, is a homolog of the spinach CSP41a, which is involved in chloroplast gene expression. BN-PAGE and RNA immunoprecipitation reveal that both *Arabidopsis* CSP41a and CSP41b are RNA-binding proteins, and prefer bind to chloroplast mRNAs rather than to rRNAs. The formation of CSP41-RNA complexes is regulated by the stromal redox state via post-translational modification. As indicated by RNA stability assay, CSP41b can stabilize chloroplast transcripts. Therefore, it can be concluded that the biological function of CSP41b is to protect RNA from degradation during the night. Such ready-to-use transcripts provide an economic way for plants rather than *de novo* RNA synthesis.

Zusammenfassung

Photosynthese ist ein biologischer Prozess, in welchem Licht und Kohlendioxid von photosynthetisierenden Organismen zu organischen Verbindungen umgewandelt wird. In Pflanzen findet Photosynthese in Chloroplasten statt, Organellen welche sich aus der Endosymbiose eines cyanobakteriellen Vorfahren ableiten und entwickelten. Das Chloroplastenproteom beinhaltet tausende Proteine, welche vorwiegend im nukleären Genom kodiert sind. Transkriptom Analysen zeigten, dass die Expression von Genen die für Proteine der Photosynthese und der plastidären Genexpression kodieren, auf transkriptioneller Ebene co-reguliert werden. Um diese Hypothese zu testen, wurden zwei co-regulierte Gene unbekannter biologischer Funktion, Putatives Photosynthetisches Protein 1 (PPP1) und 3 (PPP3) mittels reverser Genetik und biochemischer Methoden in *Arabidopsis thaliana* untersucht. In *Arabidopis* zeigen weder PPP3 knockout-Mutanten noch PPP3-eGFP-Überexpressionslinien einen distinkten Phänotypen. PPP3 ist in der Lage, einen kleinen Proteinkomplex auszubilden, welcher sich an Thylakoidlamellen anheftet. Diese Interaktionen werden vermutlich durch zwei Domänen für Protein-Protein Interaktion (TRP und PDZ) verursacht.

PPP1, bekannt als CSP41b, ist ein Homolog von CSP41a in Spinat, welches an der plastidären Genexpression beteiligt ist. BN-PAGE- und RNA Immunopräzipitationanalysen in *Arabidopsis* zeigen, dass beide Proteine, CSP41a und CSP41b RNA bindende Proteine sind, wobei sie die Bindung plastidärer mRNAs gegenüber rRNAs bevorzugt wird. Die Ausbildung der CSP41-RNA Komplexe wird über den stromalen Redoxzustand mittels posttranslationaler Modifikation reguliert. Zusätzlich konnte mit RNA Stabilitätsassays gezeigt werden, dass CSP41b plastidäre Transkripte stabilisiert. Daraus kann geschlossen werden, dass CSP41b plasidäre Transkripte während der Nacht vor Degradation schützt. Diese “ready-to-use“ Transkripte ermöglichen Pflanzen einen ökonomischeren Weg als *de novo* RNA Synthese.

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Abbreviations

°C	Degree celsius
ATP	Adenosine triphosphate
Chl	Chlorophyll
Ci	Curie
CO_2	Carbon dioxide
Co-IP	Co-immunoprecipitation
CSK	Chloroplast sensor kinase
CSP41	Chloroplast stem loop binding protein 41 kDa
cTP	Chloroplast transit peptide
Da	Dalton
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylene diamin tetraacetic acid
EF	Elongation factor
ER	Endoplasmic reticulum
g	Gram
g	Force of gravity
GFP	Green fluorescent protein
Grx	Glutaredoxin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IF	Initiation factor
LEF	Linear electron flow
LHC	Light harvesting complex
M	Mole(s) per litre
min	Minute
mol	Mole
mRNA	Messenger RNA
MS	Mass spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate
NDH	NAD(P)H dehydrogenase complex
OEC	Oxygen Evolving Complex

PAGE	Polyacrylamide gel electrophoresis
PC	Plastocyanin
p*I*	Iso-electric point
PPP1/3	Putative photosynthetic protein 1/3
PQ	Plastoquinone
PQH_2	Plastohydroquinone
PSI	Photosystem I
PSII	Photosystem II
PVDF	Polyvinylidene difluoride
RF	release factor
RIP	RNA Immunoprecipitation
RNA	Ribonucleic acid
RNAP	RNA polymerases
rRNA	Ribosomal RNA
RuBisCo	Ribulose-1,5-bisphosphate carboxylase oxygenase
S	Sedimentation coefficient
SDR	Short chain dehydrogenase/reductase
SDS	Sodium dodecyl sulphate
TPR	Tetratrico Peptide Repeat
Tris	Tris(hydroxymethyl)-aminomethane
tRNA	Transfer RNA
Trx	Thioredoxin
UTR	Untranslated region
w/v	Weight per volume
WT	Wild type
β-DM	ß-dodecylmaltoside
µ	Micro

1 Introduction

1.1 Photosynthesis

Photosynthesis is the conversion of light energy and carbon dioxide to organic compounds (primarily sugars), hereby releasing oxygen. Photosynthetic organisms are designated as photoautotrophs, and include plants, green algae (Eukaryotes) and cyanobacteria (Prokaryotes). Photosynthesis of eukaryotes takes place in an organelle, named chloroplast.

Photosynthesis includes a series of biochemical reactions accomplished cooperatively by pigments and protein complexes. Firstly, chlorophyll pigments are excited to higher-energy state as Chl* by absorbing photons. The excited Chl* can either quench to the ground state by emitting fluorescence, or by transfer energy to the reaction center to drive photochemical reactions. The transferred energy is employed to split H_2O into oxygen, protons and electrons by the Oxygen Evolving Complex (OEC) attached to PSII (Photosystem II). Protons accumulated in the lumen generate a proton gradient across the thylakoid membrane, which can be used by the ATP synthase to produce ATP. Electrons which are transferred to PSI (Photosystem I) from PS II via the Cytochrome b_6f complex finally reduce $NADP^+$ to NADPH. Both ATP and NADPH are used in the Calvin-Benson Cycle to fix CO_2. The RuBisCo (Ribulose-1,5-bisphosphate carboxylase oxygenase) complex plays a key role in the first step of carbon fixation.

The steps mentioned above are a brief description of the linear electron flow (LEF) occurring on the thylakoid membrane (Figure 1.1), which contains several photosynthetic complexes and dozens of subunits. For instance, PSII comprises around 20 subunits (Guskov *et al.*, 2009), $Cytb_6f$ (Cytochrome b_6f complex) 8 subunits (Lennartz *et al.*, 2001), PSI 21 subunits (Zolla *et al.*, 2007), and the ATP synthase 9 subunits (McCarty *et al.*, 2000).

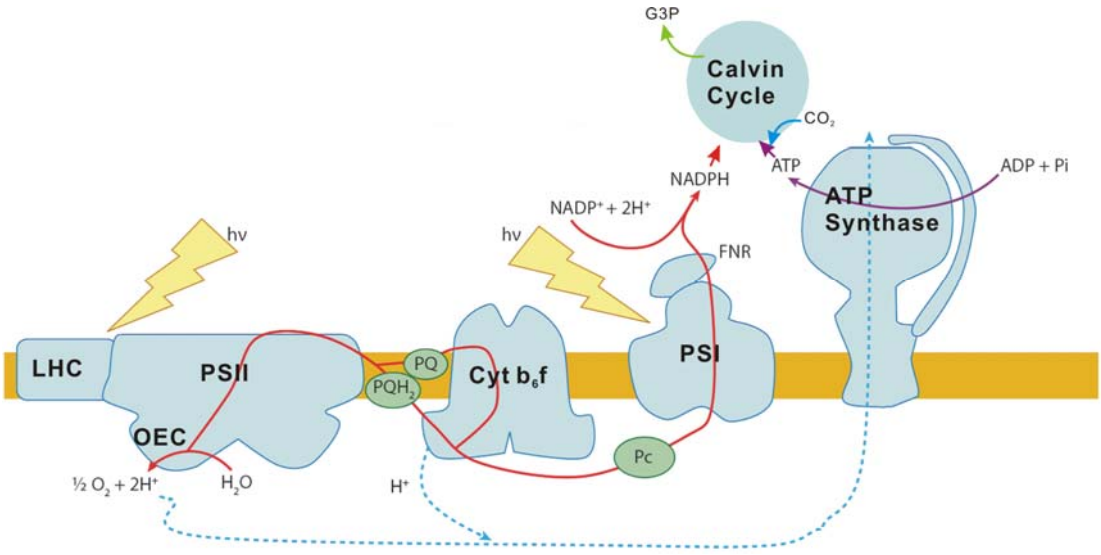


Figure 1.1 Linear electron transfer flow (LEF), modified based on the Figure from Eberhard *et al.*, 2008

Electrons derived from H_2O are transferred from PSII to PSI by oxidizing plastohydroquinone (PQH_2) and by reducing plastocyanin (PC) via Cytochrome b_6f. NADPH and ATP generated by LEF are used for carbon fixation in the Calvin cycle.

1.2 Genes transfer during endosymbiosis of chloroplast

The first paper about endosymbiosis was published in 1905 (Mereschkowsky, 1905; Martin, 1999), and it is now well accepted that modern plant cells evolved after serial primary endosymbioses. Firstly, mitochondria were derived from proteobacteria, and secondly chloroplasts were derived from cyanobacteria (Figure 1.2, Kutschera and Niklas, 2005).

There are many evidences showing that genes were transferred into the nuclear genome from the respective endosymbiont during evolution. In the model organism for higher plants, *Arabidopsis thaliana*, an entire copy of the 367-kb mitochondrial genome (Kutschera and Niklas, 2005) can be found on chromosome 2 close to its centromere . Direct measurement of the transfer rate of chloroplast DNA into the nucleus indicates that genes transfer is a frequent event, having a significant impact on the nucleus (Huang *et al.*, 2003). Based on phylogenetic analyses, it is estimated that around 400 (1.6%) -2200 (9.2%) Arabidopsis nuclear genes originate from cyanobacteria (Rujan and Martin, 2001).

The 125-Mb *Arabidopsis thaliana* nuclear genome contains 25498 protein-coding genes (The Arabidopsis Genome Iniative, 2000), and *Synechocystis sp. PCC6803*, the model organism for cyanobacteria, has a 3.9-Mb genome containing 3725 genes, among which at least 1500 are coding for proteins (Nakao *et al.*, 2009; Raven and Allen, 2003). The estimated size of chloroplast proteomes based on cTP (chloroplast Transit Peptide) predictions is about 2100 in *Arabidopsis* (Richly and Leister, 2004). The current version of chloroplast proteome by the experimental plastid database contains 1367 known proteins (Sun *et al.*, 2009), distributed between envelope, stroma or thylakoid. Estimations of the chloroplast proteome from bioinformatical and experimental analyses suggest that approximately the same amount of proteins exists in a fully functional chloroplast and in its endosymbiotic ancestor, cyanobacteria.

However, the genome of chloroplast had been dramatically reduced in comparison to that of its endosymbiotic ancestor. For instance, the chloroplast genome of *Arabidopsis thaliana* contains 85 protein-encoding genes and 44 structural RNAs (Sato *et al.*, 1999). The rest of the chloroplastic proteins are encoded by the nuclear genome, translated in cytosol, and imported into the organelle (Jarvis and Soll, 2002).

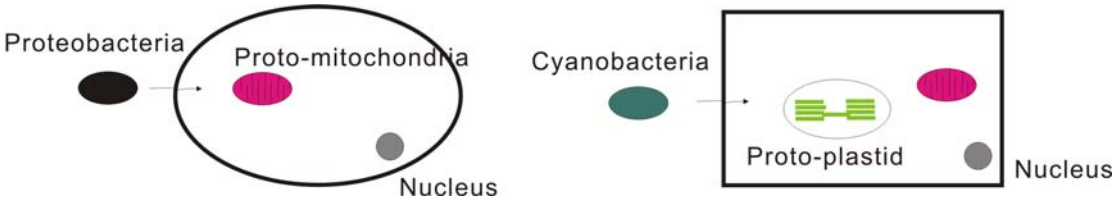


Figure 1.2 Origin of chloroplast by endosymbiosis, modified based on the Figure from Kutschera and Niklas, 2005

1.3 Chloroplast gene expression

During endosymbiosis, most of the genes from the cyanobacterial ancestor were transferred into the nuclear genome, but the rest of the plastid genome retains a set of genes for transcription (RNA polymerase subunits), translation (ribosomal proteins, rRNAs and tRNAs), photosynthesis (subunits for PSII, PSI, $Cytb_6f$ and ATP synthase, NDH complexes and Rubisco) and other functions (Sato *et al.*, 1999).

Chloroplast genes are typically organized as operons, which are transcribed as polycistronic RNAs before they are processed into single RNA units (Westhoff and Herrmann, 1988; Barkan, 1988). Two distinct RNA polymerases (RNAP), the plastid-encoded (PEP) and the nuclear-encoded (NEP) RNA polymerase are involved in chloroplastic transcription. NEP of *Arabidopsis* is a single subunit similar to mitochondrial RNAP of yeast, bacteriophages T7, T3 and SP6 (Hedtke *et al.*, 1997; Bruce Cahoon and Stern, 2001). NEP can recognize promoters with conserved YRT motives immediately upstream of the transcription initiation site, but not in all cases (Weihe and Borner, 1999). The core PEP in chloroplasts is composed of 4 subunits encoded by chloroplast genes (*rpoA, rpoB, rpoC1* and *rpoC2*), and is activated by adding sigma-like transcriptional factors (SLF) for recognition of promoters (Little and Hallick, 1988; Isono *et al.*, 1997; Suzuki *et al.*, 2004). The activated PEP complex contains several accessory proteins encoded by nuclear genes (Pfalz *et al.*, 2006; Suzuki *et al.*, 2004; Pfannschmidt *et al.*, 2000), which shows that the nuclear genome has a striking impact on the regulation of chloroplast genome transcription. It is thought that Both RNA polymerases act cooperatively in plastid transcription. NEP is responsible for transcribing plastid genetic machinery genes and PEP genes, whereas PEP is mainly responsible for transcribing photosynthesis-related genes (Lopez-Juez and Pyke, 2005).

The translation apparatus in chloroplasts (70S ribosome that contains 23S, 16S and 5S rRNAs) is similar to that of prokaryotes, but different from the cytosolic 80S ribosome (Trempe and Glitz, 1981; Manuell *et al.*, 2007). The plastid ribosome of spinach comprises 59 proteins (33 for 50S subunit, 25 for 30S subunit and 1 ribosome recycling factor), among which 53 are orthologs of *Escherichia coli* ribosomal proteins and 6 are plastid-specific proteins (Yamaguchi and Subramanian, 2000). In *Chlamydomonas*, two additional proteins, RAP38 and RAP41, similar to CSP41 in spinach, were found in the 70S ribosome, but neither in the 50S nor in the 30S subunit (Yamaguchi *et al.*, 2003). Orthologs of general translation factors required for initiation (IF1, IF2 and IF3), elongation (EF-Tu, EF-Ts, EF-G) and release/recycling (RF1, RF2, RF3) in prokaryotes were found to be encoded by the genome of cyanobacteria, green algae, as well as vascular plants (Marin-Navarro *et al.*, 2007; Beligni *et al.*, 2004; Harris *et al.*, 1994). Shine-Dalgarno-like sequences, the binding

site to 16S rRNA of 30S subunit for translation initiation, are present in the 5' untranslated region (UTR) of many but not all chloroplast mRNAs (Hirose and Sugiura, 2004; Harris *et al.*, 1994; Ruf and Kössel, 1988).

1.4 Regulation of Chloroplast gene expression

Since the organelles evolved from a cyanobacterial ancestor, chloroplasts have prokaryotic and eukaryotic features of gene expression. The transcription and translation apparatus in chloroplasts require a large number of nucleus-encoded factors, which provide several regulatory levels of chloroplast gene expression. Besides regulation mechanisms on the transcriptional and the translational level, two other regulation pathways of chloroplast gene expression are possible; the post-translational RNA processing and the post-translational modification level (Figure 1.3, blue arrows).

1.4.1 Transcriptional regulation

Some chloroplast transcript levels show peaks at the early stage of chloroplast biogenesis. In barley, RNAs, which encode for subunits of PEP (*rpoB*, *rpoC1* and *rpoC2*) and *rps16,* reach maximal abundance in chloroplasts development prior to genes encoding for subunits of photosynthetic complexes (*rbcL, atpB, psaA* and *petB*) (Baumgartner *et al.*, 1993). *psbD-psbC* transcripts accumulation in barley is induced by blue light, but neither by red nor by far-red light (Gamble and Mullet, 1989). In the mature chloroplast, the redox state of plastoquinone controls the transcription rates of genes encoding for the reaction center of PSI and PSII (Pfannschmidt *et al.*, 1999). Two kinases, STN7 (on thylakoid membranes) and CSK (in the stroma) in *Arabidopsis* chloroplasts are thought to connect the redox state of thylakoid membranes and chloroplast gene transcription (Bonardi *et al.*, 2005; Puthiyaveetil *et al.*, 2008; Pesaresi *et al.*, 2009).

1.4.2 Post-transcriptional regulation

Regulation of chloroplast gene expression also relies on post-transcriptional processing of chloroplast primary transcripts, including 5'- and 3'-end processing,

intercistronic cleavage, 5'- and 3'-end maturation and RNA editing (del Campo, 2009; Stern *et al.*, 2010). These processes require several nucleus-encoded factors, such as endoribonucleases, exonucleases and numerous RNA-binding proteins. In *Arabidopsis*, a chloroplast RNase E (endoribonuclease) knock-out mutant shows overaccumulation of polycistronic precursor transcripts (Walter *et al.*, 2010). A huge RNA-binding protein family, annotated as PPR (Pentatricopeptide Repeat), contains 450 members in *Arabidopsis*. PPR family proteins play an important role in organellar post-transcriptional processing (O'Toole *et al.*, 2008). In *Arabidopsis*, HCF152 with 12 putative PPR motifs is involved in *psbB-psbT-psbH-petB-petD* transcript processing (Meierhoff *et al.*, 2003). PPR10 in maize binds to a specific sequence motif in *atpI-atpH* or *psaJ-rpl33* mRNA, as a barrier to RNA degradation from either the 5' or 3' direction (Pfalz *et al.*, 2009; Barkan, 2011). During editing of higher plant mRNA, cytosine (C) is converted to uracil (U) occurring in the organelle. CRR4 of *Arabidopsis* is another sequence-specific chloroplast PPR protein involved in creating the translational initiation codon of the plastid-encoded *ndhD* gene (Kotera *et al.*, 2005; Oduka *et al.*, 2006).

1.4.3 Translational regulation

In Chlamydomonas, the abundance of some chloroplast transcripts (*psaB*) fluctuates between dark and light, but some (*psbA, rbcL*) remain constant (Salvador *et al.*, 1993). For the latter that do not fluctuate, regulation of gene expression occurs mainly at the translational level. Translational regulation of chloroplast genes is mainly taken place at the translation initiation level, which is mediated by direct or indirect interaction of nucleus-encoded factors with *cis*-elements mainly at the 5' UTR of chloroplast mRNAs (Marin-Navarro *et al.*, 2007; Barnes *et al.*, 2005; Katz and Danon, 2002). One of the RNA-binding proteins, RB47, which is necessary for translational initiation of *psbA* mRNA, is regulated by the redox state via a protein disulfide isomerase, RB60 (Marin-Navarro *et al.*, 2007; Kim and Mayfield, 1997; Alergand *et al.*, 2006).

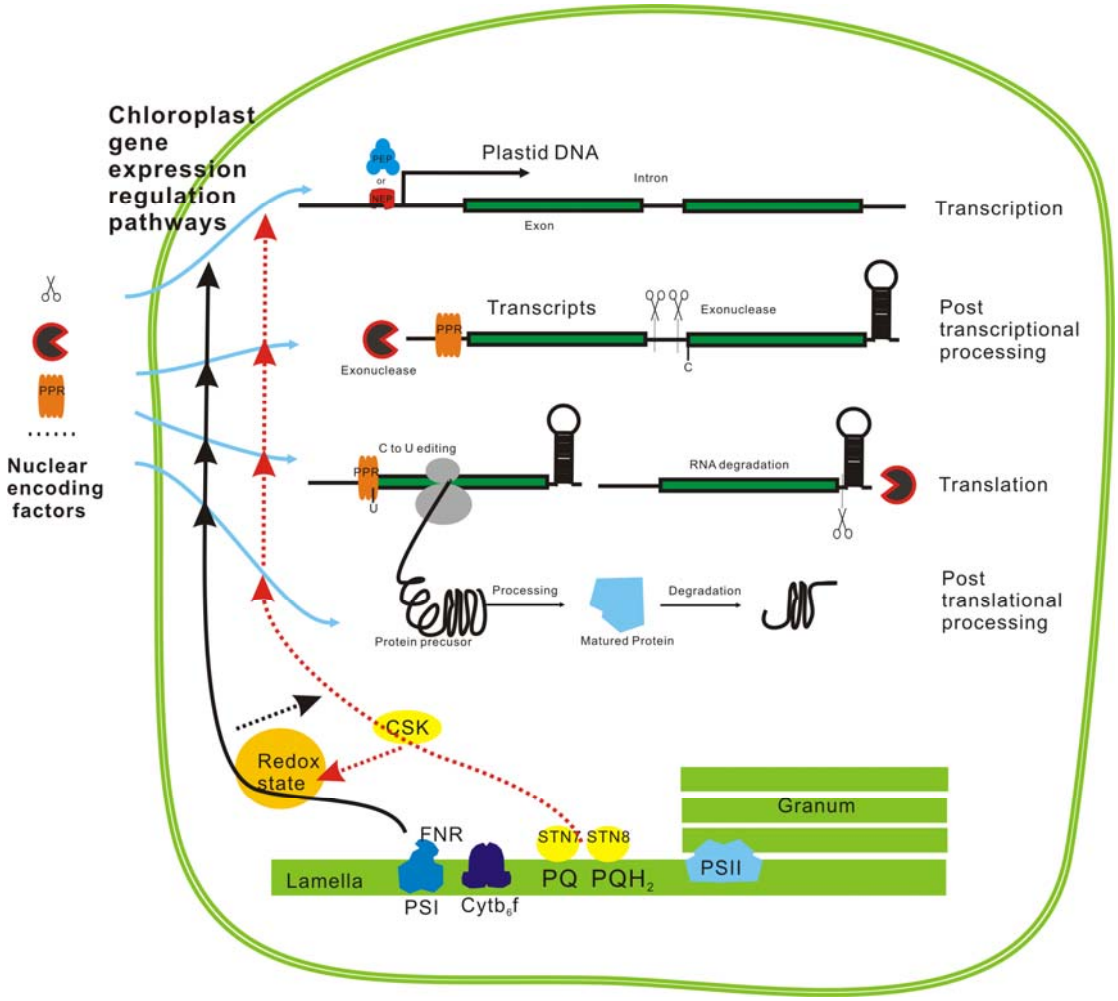


Figure 1.3 Regulation of chloroplast gene expression

Chloroplast gene expression can be regulated at different levels, such as the transcriptional level, post-transcriptional level, translational level and post-translational level. These regulatory pathways involve several factors that are translated in the cytosol, and imported into the chloroplast as shown by blue arrows. Thylakoid redox signals could also regulate proteins from both nucleus and chloroplast via post-translational modifications, such as phosphorylation (red arrow) and disulphide bond formation (black arrow).

1.4.4 Post-translational regulation

Generally, the biological activity of a nascent protein is not only dependent on its primary sequence, but also on post-translational modifications (Tsou, 1988; Warzecha, 2008). Although it is common for chloroplast proteins to be subjected to post-translational modifications, such as phosphorylation (Reiland *et al.*, 2009), acetylation

(Finkemeier *et al.*, 2011) and disulphide bridge formation (Stroher and Dietz, 2008), only modifications of chloroplast-encoded proteins will be discussed here as post-translational regulation mechanism. Modification to nucleus-encoded chloroplast proteins which are involved in transcription or translational processes is considered as transcriptional or translational regulation, such as disulphide bridge formation for RB47 in part 1.4.3. Post-translational processing of the nascent D1 (*psbA*) precursor to mature D1 is essential to establish the activated PSII complex (Anbudurai *et al.*, 1994; Wei *et al.*, 2010). By using mass spectrometry, a highly conserved amino acid Trp-352 of CP43 (light harvesting protein) with multiple types of modifications were detected, and these modifications could play a role in turnover of PSII reaction center (Anderson *et al.*, 2002). Degradation of the large subunit of RuBisCo and D1 photo-damaged protein depends on post-translational modifications (Desimone *et al.*, 1998; Koivuniemi *et al.*, 1995; Aro *et al.*, 1993).

1.5 Redox regulation of chloroplast gene expression

Plants have to cope with fluctuating conditions, such as changes in diurnal rhythm, temperature and light intensity. Environmental acclimation can be divided into short-term (from seconds to minutes) and long-term (from hours to days) responses. The mechanisms of acclimation, such as state transitions (short-term acclimation) and regulation of chloroplast gene expression (long-term acclimation), are connected closely to changes of the redox state of the plastoquinone pool (Dietzel *et al.*, 2008; Eberhard *et al.*, 2008; Rochaix, 2011).

Regulation of chloroplast gene expression via redox states generated by photosynthesis is a rather complicated network shown as nodes crossed blue arrows to red and black arrow (Figure 1.3). Firstly, the chloroplast gene expression at four different levels (Fig 1.3 blue arrow; Part 1.4) is controlled by hundreds of nucleus-encoded factors which could be regulated by their own promoters. Secondly, when these factors have been imported into the chloroplast, they could be regulated by post-translational modification via disulphide bonds (-S-S-) or phosphorylation (Figure 1.3, red and black arrow).

Redox regulation via disulphide bridges was discovered during studies on chloroplast enzymes (Wolosiuk and Buchanan, 1977; Buchanan, 1980). For instance, an enzyme in the Calvin cycle, fructose-1,6-bisphosphatase (FBPase), is light regulated via a disulphide bond by thioredoxin (Trx) in the chloroplast (Clancey and Gilbert, 1987; Buchanan, 1991; Balmer and Schurmann, 2001). The plant genome encodes numerous Trxs and Grxs (Glutaredoxin), which could regulate proteins involved in chloroplast metabolism and in gene expression (Buchanan and Balmer, 2005; Meyer *et al.*, 2009). Combining diagonal PAGE and mass spectrometry analayses for the identification of the thiol-disulphide redox proteome reveal that 22 novel chloroplast proteins are putative targets of redox regulation in photosynthesis, metabolism and chloroplast gene expression (Ströher and Dietz, 2008). Possible target proteins of Trx can be found with a mutated Trx (m and f type) in which an internal cysteine at the activated site is substituted by serine. Via this approach, 11 previously unknown and 9 confirmed targets were identified (Motohashi *et al.*, 2001; Balmer *et al.*, 2003). By affinity chromatography, proteins associated with Trx by electrostatic force were analyzed (Balmer *et al.*, 2004). Some putative targets involved in chloroplast gene expression were discovered in different experiments; for instance (i) RB60, 28 kDa ribonucleoprotein, elongation factor Tu, elongation factor g and ribosomal proteins (S1, S5, S30, L4, L21) acting on the translational level (Balmer *et al.*, 2003; Balmer *et al.*, 2004, Alergand *et al.*, 2006); (ii), two RNA-binding proteins (homologs to CP31 and CSP41a) acting on the post-transcriptional level (Balmer *et al.*, 2004; Stern *et al.*, 2010; Ströher and Dietz, 2008; Tillich *et al.*, 2009); (iii), two transcription factors (Glabra2 and Why3) acting on the transcriptional level (Ströher and Dietz, 2008); (iv), ATP dependent Clp protease and Hsp70 acting on the post-translational level (Balmer *et al.*, 2003; Balmer *et al.*, 2004; Ströher and Dietz, 2008).

1.6 Chloroplast phosphoproteome and chloroplast gene expression.

Phosphorylation is one of the most important post-translational modifications to activate or deactivate proteins in cells. Although there is no plastome-encoded kinase or phosphatase, the chloroplast phosphoproteome contains at least 174 phosphoproteins (Reiland *et al.*, 2009), among which a portion is involved in chloroplast gene expression (e.g. ribosomal proteins, subunits of NEP and RNA-binding proteins). In Arabidopsis, at least two thylakoid membrane associated kinases,

STN7 and STN8, are known. Their substrates are photosynthetic subunits, but no stromal protein targets for STN7 and STN8 are known in plants (Bonardi *et al.*, 2005; Pesaresi *et al.*, 2010). The requirement of STN8 for phosphorylation of lammellae-localized CaS (calcium-sensing receptor) suggests a possible signaling pathway to stromal processes (Vainonen *et al.*, 2008, Pesaresi *et al.*, 2010). Phosphorylation cascades initiated from the redox state of plastoquinone pool may require soluble kinase in the stroma, such as CSK and CKII (Casein Kinase II), to regulate chloroplast gene expression (Baginsky and Gruissem, 2009, Puthiyaveetil *et al.*, 2008). One of the direct protein substrate of redox-sensitive CKII, in *Arabidopsis*, AtSIG6, may function as a general sigma factor in early plant development (Schweer *et al.*, 2010; Ogrzewalla *et al.*, 2002; Ishizaki *et al.*, 2005). The substrates of CSK are not known, yet. But it (?) provides a redox regulatory mechanism that connects photosynthesis to chloroplast gene expression (Puthiyaveetil *et al.*, 2008). Since kinases or phosphatases themselves could be regulated by redox or auto-phosphorylation, it seems that chloroplast gene expression is regulated rather by crossed pathways than by two independent parallel cascades (Figure 1.3).

1.7 Localization of chloroplast gene translation

Polysomes and mRNAs bound to thylakoid membranes raise to the question ‘where chloroplast translation occurs’. It was hypothesized that membrane proteins were synthesized by membrane-bound polysomes and soluble proteins by stroma-localized polysomes (Margulies, 1983; Jagendorf and Michaels, 1990). However, soluble proteins such as RbcL and elongation factor EF-Tu were also found to be translated by membrane bound polysomes (Hattori and Margulies, 1986; Muhlbauer and Eichacker, 1999; Breidenbach *et al.*, 1990). Three models about this question were proposed: (i), chloroplast mRNAs are translated in association with thylakoid membranes; (ii), chloroplast mRNAs are translated in association with the inner membrane of the chloroplast envelope; (iii), chloroplast mRNAs are translated at the end of thylakoid grana, the stacks of disk-like thylakoid membranes (Zerges, 2000). Ribosomal proteins, RNA-binding proteins and elongation factors were found on thylakoid membranes by proteome analysis, showing that the thylakoid surface is an important site for protein synthesis (Friso *et al.*, 2004; Peilter *et al.*, 2004).

Translation targeting on thylakoid membranes in chloroplasts shares similarities to targeting on endoplasmic reticulum membrane (ER). It could be shown that the chloroplast homolog cpSRP54 (Signal Recognition Particles to drive ribosome-mRNA-nascent peptide to ER) is associated with the nascent D1 subunit of PSII and the 70S ribosome (Eichacker and Henry, 2001; Richter *et al.*, 2010). The mRNA-based targeting mechanism requires RNA-binding proteins (RBP) to transfer mRNA onto ER for translation initiation (Johnston, 2005). This is supported by the fact that several chloroplast RBP were found to be associated with thylakoid membranes (Uniacke and Zerges, 2009; Friso *et al.*, 2004). For that reason, it can be suggested that the translation apparatus is directly regulated via phosphorylation by thylakoid-associated kinases or phosphatases.

1.8 Novel putative photosynthetic proteins

As it has been discussed in part 1.2 (Gene transfer during endosymbiosis of chloroplast), the predicted number of chloroplast proteins is around 3000 in Arabidopsis. Over the past few decades, knowledge about protein function in chloroplast biogenesis, gene expression and photosynthesis has been accumulated, but there are still many chloroplast proteins with unknown or ambiguous function. The mRNA expression of 3292 nuclear genes, most of which encode for chloroplast-localized proteins (Richly and Leister, 2004) were determined under a total of 101 different environmental and genetic conditions (Biehl *et al.*, 2005). This analysis showed that 1590 most-regulated genes fell into 23 distinct groups of co-regulated genes (regulon). The co-expressed nuclear genes in regulon 1 encode mainly for subunits of photosystems. Regulon 2 comprises genes involved in the transcription/translation of plastome genes (particularly ribosome polypeptides). Two of those proteins being co-regulated with photosynthetic genes have been named PPP1 and PPP3 (Putative Photosynthetic Protein 1 and 3) and are the subjects of this thesis.

1.9 Chloroplast mRNA-binding proteins

In chloroplasts, there are abundant RNA-binding proteins which could regulate chloroplast gene expression. PPR family proteins are involved in mRNA metabolism

by recognizing specific RNA sequences (Pfalz *et al.*, 2009; Stern *et al.*, 2010). cpRNP (chloroplast ribonucleoprotein) family proteins employ two RNA-recognition motifs and an acidic N-terminal domain to bind various chloroplast mRNAs (Nakamura *et al.*, 2004; Tillich *et al.*, 2009). It has been reported that another chloroplast mRNA-binding protein family, such as CSP41 binds to the stem-loop structure in the 3' UTR region of mRNA. Therefore, it is suggested that this protein family is involved in stabilizing mRNA and in forming correct 3'-end (Stern *et al.*, 2010).

One of the subjects in this thesis, PPP1 also known as CSP41b, is a homolog to CSP41a in *Arabidopsis*. CSP41a was pulled down by a heparin-agarose column in assaysusing spinach. CSP41acould bind the stem-loop structure in the 3' UTR region of *petD* mRNA (Chen *et al.*, 1995). Heterologously expressed CSP41a from *E.coli* shows a non-specific RNA endoribonuclease activity (Yang *et al.*, 1996). However, the fact that the RNA-binding activity affects the rate of RNase activity suggests an additional role of CSP41a in the chloroplast RNA mebabolism (Yang and Stern, 1997). CSP41 proteins were also tentatively identified as components of the plastid-encoded RNA polymerase (PEP) in mustard (Pfannschmidt *et al.*, 2000), but not in later proteome analysis of the PEP complex (Suzuki *et al.*, 2004; Pfalz *et al.*, 2006). In Chlamydomonas, CSP41a and CSP41b were identified as a component of the 70S ribosome, but not found in either of the ribosomal subunits by MS sequencing of purified ribosome (Yamaguchi *et al.*, 2003). Those results are supported by different research groups to some extent. For instance, CSP41a and CSP41b can form different molecular size complexes detected by CN-PAGE of the stroma and subsequent MS analysis: (i) in a complex larger than 950 kDa most likely associated with 70 S ribosomes, (ii) at 224 kDa and (iii) at 106–126 kDa (Peltier *et al.*, 2006), but neither CSP41a nor CSP41b co-migrated with mature chloroplast ribosomes to detectable levels (Beligni and Mayfiled, 2008). The low molecular complex could be caused by the dimerization of CSP41a and CSP41b, which was supported by using Flag-tagged CSP41b to pull down CSP41a, and depletion of CSP41a in mature leaves of the *csp41b* mutant (Bollenbach *et al.*, 2009).

Reverse genetic approaches were used to knock out CSP41 to analyze its biological function. Early CSP41a RNAi depletion lines of tobacco show no difference compared to the wild type (Bollenbach *et al.*, 2003). *csp41a* and *csp41b* single mutant

of *Arabidopsis* show no distinct phenotype, but the double mutant *csp41a*csp41b* is lethal (Beligni and Mayfiled, 2008). However, the *csp41b* mutant of Arabidopsis in some research groups show different phenotypes, such as altered chloroplast morphology, photosynthetic performance and circadian rhythm (Hassidim *et al.*, 2007), pale green leaves and reduced accumulation of the ATP synthase and cytochrome b_6f complexes (Bollenbach *et al.*, 2009).

1.10 TPR proteins in higher plants

TPR proteins are a large number of proteins containing TPR (Tetratrico Peptide Repeats) motif composed of 3-16 tandem repeats of 34 amino acid residues firstly discovered in the 1990s (Hirano *et al.*, 1990; Sikorski *et al.*, 1990). Unlike PPR proteins, which evolved from TPR and mainly function in organelle transcripts processing in eukaryotes, TPR proteins usually mediate protein-protein interactions and the assembly of multiprotein complexes (Small and Peeters, 2000; D'Andrea and Regan, 2003; Lurin *et al.*, 2004). TPR protein YCf3 which is conserved in cyanobacteria, algae, and plants, is essential for the accumulation of the PSI complex at post-translational level (Naver *et al.*, 2001). The TPR domain of TOC64 on the envelope membrane provides a docking site for the recruitment of chaperone HSP90, which is an important step in targeting protein precursor from the cytosol into organelle (Qbadou *et al.*, 2006). REP27, a chloroplast-targeted protein containing two TPR motives, plays a dual role in regulation of D1 turnover by facilitating co-translational biosynthesis insertion and activation of the nascent D1 during the PSII repair process (Dewez *et al.*, 2009). Several TPR proteins were found to be essential for the response to hormones, such as abscisic acid (ABA), cytokinin, gibberellin and auxin in *Arabidopsis* (Schapire *et al.*, 2006).

PPP3, one of the subjects in this thesis, is a protein containing a TPR-like domain, as well as a PDZ-like domain. The PDZ domain is another protein-protein interaction domain existing in both prokaryotes and eukaryotes (Ponting, 1997). There are no more information about the biological function of PPP3 than that it is a component of the thylakoid membrane (Peltier *et al.*, 2004) and is phosphorylated in response to wounding (Ishikawa *et al.*, 2005).

1.11 Aim of this thesis

In this thesis, two putative photosynthetic proteins (PPP1 and PPP3) identified by its transcriptional co-regulation with known photosynthetic genes, were characterized by reverse genetic approaches. The first aim was to clarify if PPP3 is a thylakoid membrane attached protein, and if it is a part of any photosynthetic complexes. The second aim was to prove CSP41a and CSP41b to be RNA-binding proteins *in vivo* with different RNA-binding preferences. The third aim was to study how CSP41-RNA complexes are regulated via disulphide bond or phosphorylation, and to find out if they exhibit different biological function in the chloroplast. Finally, combining all the experimental information, a model about how the CSP41-RNA complex is regulated during dark and light was proposed.

2 Materials and Methods

2.1 Plant materials and growth conditions

Three independent insertion lines of *PPP3* (At1g55480) were isolated from different ecotypes background of *Arabidopsis*. *ppp3-1* (Nössen, 12-2346-1) is a transposon (*Ds*) insertion mutant from Riken (Kuromori *et al.*, 2004). *ppp3-2* (Columbia) is a T-DNA (*PCV6NF*) insertion mutant isolated from the collection of Csaba Koncz (Rios *et al.*, 2002). *ppp3-3* (Landsberg *erecta*) was a transposon (*Ds*) insertion mutant from the Cold Spring Harbor Laboratory (http://genetrap.cshl.edu/, GT4125). Mutant lines of *CSP41a* (At3g63140) and *CSP41b* (At1g09340) were obtained from Dr. Ute Armbruster (Leister, University of Munich).

Seeds from wild type and mutant plants were stratified on water soaked Whatman paper in Petri dishes at 4°C in the dark for 3 days before sowing on soil. Plants were grown on soil in the climate chamber (PFD: 80, 120, or 500 μmol $m^{-2}s^{-1}$, 12 h/12 h dark/light) or under controlled greenhouse conditions (daylight supplemented with HQI Powerstar 400 W/D with ~180 μmol photons m^{-2} s^{-1}, 10 h/14 h dark/light). Wuxal Super (8% N, 8% P_2O_5, 6% K_2O; Manna Fertilizers, Germany) was used as fertilizer, according to the manufacturer's instructions. Leaf area was calculated from at least 20, 3-week-old plants using the software ImageJ (Abramoff *et al.*, 2004).

For plants grown on MS (Murashige-Skoog, Duchefa) plates, seeds were surface sterilised with 7.5% (v/v) hypochloride and 0.5% (v/v) Triton-X and grown on MS plates containing 1% sucrose. For stress treatments to *ppp3* mutants, 100 mM NaCl (Salt stress) or 100 mM Mannitol (Drought stress) was added into MS medium. After stratification for 4 days at 4°C, the plates were transferred to 16 h/ 8 h light dark cycle at 22°C.

2.2 Nucleic acid analyses

Arabidopsis genomic DNA was isolated by disruption of leaf material in liquid nitrogen with metal beads and addition of DNA Extraction Buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA and 0.5% SDS). After centrifugation at 16000

g for 10 min, DNA in the supernatant was precipitated by addition of 0.8 volumes of isopropanol, washed with 70% ethanol, and dissolved into 200 µl double distilled H_20 containing 20 µg/ml RNase A. 1 µl the genomic DNA was used as templates for a 10 ul-volume PCR. T-DNA and Transposon insertion junction sites were determined by sequencing of PCR products generated with combinations of insertion and gene specific primers. To genotype the *ppp3.1* and *ppp3.3* mutant, primers of *Ds3´-1, At5g55480-1s, At5g55480-371as, At5g55480-951s* and *At5g55480-1800as* were used. Primers of *Fish1*, *At5g55480-1s* and *At5g55480-371as* were used to genotype the *ppp3.2* mutant.

Total RNA was extracted from leaf materials using the TRIzol reagent (Invitrogen), according to the manufacturer's instructions. The procedures for Northern blot and probe labelling were performed generally as described by Molecular Cloning (Third Edition) (Sambrook and Russell, 2001). In brief, total RNA separated on 1.5% denaturing formaldehyde agarose gel, was transfered to Hybond N^+ membranes (GE healthcare) followed by UV light cross-linking (Stratalinker® UV Crosslinker 1800). The membrane was pre-hybridized with Pre-hybridized Buffer (7% SDS and 0.25 M Na_2HPO_4-NaH_2PO_4 pH 7.0) at least 3 hours at 65°C before adding radioactive labeled probes. After hybridization at 65°C over night, the membrane was washed twice at 60°C (1^{st} for 30 min and 2^{nd} for 15 min) with Northern Washing Buffer (2xSSPE and 0.1% SDS). After exposuring the blotted membrane to the Storage Phosphor Screen (Fuji), Signals were scanned and quantified by the Typhoon phosphorImager (GE Healthcare).

300 ng of PCR amplified DNA fragment was radioactively labeled in a 50 µl-volume reaction containing 1xOLB Buffer (50 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 0.07% (v/v) β-mercaptoethanol, 4 mM each dGTP, dATP and dTTP, 0.2 mM HEPES pH 6.0 and 0.11 mg/mL random hexamers), 10 units Klenow and 12.5 µCi [α-^{32}P] dCTP at 37°C for 3 hours. The ^{32}P labeled probes were purified by illustra MicroSpin™ G-50 Columns (GE healthcare), and denatured at 100°C for 5 min prior to the Northern hybridization.

2.3 Point mutation and Plant transformation

By PCR-based mutagenesis (Atanassov *et al.*, 2009), primers containing the mutated site and the gateway recombination site were used to generate the Cysteine-to-Serine exchanged CSP41a and CSP41b. Using the Gateway cloning technology (Invitrogen), the coding region of *PPP3* (generated by primers of *ppp3-F pdnr* and *ppp3-R pdnr*), *CSP41a* (generated by *csp41a pdnr s* and *csp41a pdnr as*), *CSP41b* (generated by *csp41b pdnr s* and *csp41b pdnr as*), *CSP41b-C161S* (generated by *csp41b C161S s*, *csp41b C161S as*, *csp41b pdnr s* and *csp41b pdnr as*), *CSP41b-C176S* (generated by *csp41b C176S s*, *csp41b C176S as*, *csp41b pdnr s* and *csp41b pdnr as*), and *CSP41b-C288S* (generated by *csp41b C288S s*, *csp41b C288S as*, *csp41b pdnr s* and *csp41b pdnr as*) was inserted into the destination vector pB7FWG2.0 (Karimi *et al.*, 2002), which can express the inserted gene fused with an eGFP (enhanced green fluorescent protein) tag at the C-terminal, under the transcriptional control of doubled Cauliflower Mosaic Virus 35S promoters. The coding region of *CSP41a-C247S* (generated by *csp41a C247S s*, *csp41a C247S as*, *csp41a pdnr s* and *csp41a pdnr as*), *CSP41a-C300S* (generated by *csp41a C300S s*, *csp41a C300S as*, *csp41a pdnr s* and *csp41a pdnr as*)) and *CSP41a-C315S* (generated by *csp41a C315S s*, *csp41a C315S as*, *csp41a pdnr s* and *csp41a pdnr as*) were inserted into the destination vector pJan33 (Weigel *et al.*, 2003).

Flowers of 30-day-old *Arabidopsis* plants were dipped into *Agrobacterium* suspensions (strain GV3101, carrying the respective destination vector) containing 2.5% sucrose and the surfactant Silwet L-77 (0.02%) for 1 min (Clough and Bent, 1998). After dipping, plants were covered with sterile plastic bags for two days to sustain high humidity levels. Afterwards, plants were transferred to the greenhouse and seeds were collected after approximately 3 weeks. Harvested seeds were selected either against BASTA (glufosinate ammonium) (for pB7FWG2.0) on soil or against 50 μg/ml Kanamycin (for pJan33) on MS plates. The selected positive line was confirmed by Western blot analysis and PCR on the genomic DNA.

2.4 Chloroplast isolation and fractionation

Leaves of 3-week-old dark adapted 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 of Miracloth (Calbiochem), and the filtrate was centrifuged at 4°C and 300 *g* for 4 min with the 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 and no break) through a two step Percoll gradient (40%-80% (v/v) in 1xResuspension Buffer) with the JS13-1 rotor at 4°C and 3250 *g* for 20 min. Intact chloroplasts were collected at the interface of the percoll gradient, and washed once by the Resuspension Buffer with the JS13-1 rotor at 4°C and 1000 *g* for 4 min.

For chloroplast fractionation, 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°C and 16000 *g* for 60 min. The supernatant (stroma fraction) was collected, and the protein concentration was measured by Bradford Protein Assay, according to the manufacturer's instructions (Biorad). The pellet (thylakoid membrane fraction) was washed twice with the TMK Buffer (10 mM pH 6.8 Tris-HCl, 10 mM $MgCl_2$ and 20 mM KCl). Intact chloroplasts, the thylakoid membrane fraction and the stroma fraction equivalent to equal amount of chlorophyll were resolved on a 15% SDS-PAGE gel for immunoblot assays.

2.5 Immunoblot assays

Proteins separated by SDS-PAGE were transferred to PVDF membranes (Millipore) by a semi-dry blotting system (Biorad) using a current corresponding to 1 mA cm^{-2} with Towbin Buffer (96 mM Glycine, 10 mM Tris and 10% (v/v) methanol) (Towbin *et al.*, 1979). Proteins were visualized by staining the PVDF membrane with Coomassie Solution (0.02% Coomassie R 250 in 50% methanol). The PVDF membrane was incubated with specific primary antibodies for 2 hours at room temperature, followed by washing with TBST (1xTBS and 0.1% Tween-20) 3 times (10 min each). The washed membrane was probed with secondary antibodies (ECL

Rabbit IgG, HRP-Linked Whole antibody from donkey, Amersham) at room temperature for 1 hour. After washing with TBST 3 times (10 min each), signals were detected by enhanced chemiluminescence solution (ECL Kit, Amersham Biosciences) using an ECL reader (the Fusion FX7, PeqLab).

2.6 Thylakoid fractionation and Salt treatment

Intacted chloroplasts (from Part 2.4) were lysed in Lysis Buffer (25 mM HEPES-KOH pH 7.5 and 5 mM $MgCl_2$) for thylakoid preparation. Thylakoids were solubilized in Fractionation Buffer (15 mM Tricine-KOH pH 7.9, 0.1 M Sorbitol, 10 mM NaCl and 5 mM $MgCl_2$) containing 0.2% digitonin for 1 min at room temperature. The solubilization was stopped by adding 10 volumes of the Fractionation Buffer without digitonin. The short solubilization fractionated thylakoid membranes into grana, intermediate membranes and stroma lamellae. The different fractions were obtained by differential centrifugation (Ossenbuehl *et al.*, 2002). In brief, the suspension was centrifuged four times at 4 °C, and each supernatant was used for the next centrifugation step. The centrifugations were performed at 1000 *g* for 10 min, 10000 *g* for 30 min (pellet as grana), 40000 *g* for 60 min (pellet as intermediate membrane) and 150000 *g* for 90 min (pellet as stroma lamellae). The pellet of each step were collected, normalized with the chlorophyll concentration and resolved with SDS-PAGE.

Salt treatments to thylakoids were performed as published (Karnauchov *et al.*, 1997). Membranes were treated on ice for 30 min with final concentration of 2 M NaCl, 0.1 M Na_2CO_3, 2 M NaSCN and 0.1 M NaOH in HM Buffer (10 mM HEPES-KOH pH 8.0, 5 mM $MgCl_2$). The supernatant and the pellet were separated by centrifugation at 4°C and 10000 *g* for 10 min, and were resolved on Tricine-SDS-PAGE for Western blot analyses.

2.7 2D Blue Native-PAGE/Tricine-SDS-PAGE

Blue Native-PAGE (the first dimension) analysis of the thylakoid was performed as followings. Thylakoids equivalent to 30 μg (for β-DM treatment) or 50 μg (for digitonin treatment) chlorophyll were resuspended in 60 μl ACA Buffer (750 mM ε-

aminocaproic acid, 50 mM Bis-Tris-HCl pH 7.0, 5 mM pH 7.0 EDTA and 50 mM NaCl). After adding β-DM or digitonin to the final concentration of 1% or 1.7%, respectively, thylakoids were solubilized on ice for 20 min (for β-DM treatment) or 60 min (for digitonin treatment), and the suspension were centrifuged at 4°C and 16000 *g* for 20 min (β-DM treatment) or 60 min (digitonin treatment). The supernatant was mixed with 1/20 volume of BN Loading Dye (750 mM ε-aminocaproic acid and 5% Commassiee G 250) and resolved on a 4%-18% BN-PAGE gel (Acrylamide gel containing 0.5 M ε-aminocaproic acid, 20% glycerol (only in heavy gel) and 50 mM Bis-Tris-HCl pH 7.0). The BN-PAGE was performed using Cathode Buffer (50 mM Tricine, 15 mM Bis-Tris-HCl pH 7.0 and 0.02% Coomassie G 250) and Anode Buffer (50 mM Bis-Tris-HCl pH 7.0) with a constant voltage of 70 V at 4°C overnight. Gel lanes containing samples were cut off, denatured in Denaturing Buffer (0.125 M Tris-HCl pH 6.8, 4% SDS and 1% β-mercaptoethanol) for 30 min and resolved on the 2nd dimension of Tricine-SDS-PAGE as described (Schägger, 2006).

To analyse stromal proteins by Blue Native-PAGE, 150 μg stroma sample was mixed with 1/20 volume of BN Loading Dye, and the mixture was directly loaded on a 4%-18% BN-PAGE gel. The electrophoresis was performed the same as in the BN-PAGE to thylakoids, except that only 0.002% Coomassie G 250 was used in the Cathode Buffer.

2.8 *In vivo* translation assay

In vivo radioactive labeling of thylakoid proteins was performed as described (Pesaresi *et al.*, 2006). 5 leaves of Arabidopsis harvested at the 12-leaf rosette stage were pressed softly against a sandpaper, and vacuum infiltrated in a 20 ml syringe containing 1 mCi [^{35}S] Methionine, 20 μg/ml cycloheximide and 0.1% Tween-20 in 5 ml TME Buffer (20 mM Tris-HCl pH 6.8, 10 mM $MgCl_2$ and 5 mM EDTA). After infiltration, leaves were illuminated with light (50 μmol m^{-2} s^{-1}) for 30 min. Thylakoids were isolated and resolved on Tricine-SDS-PAGE. The gel was stained with Coomassie and dried on a Whatman filter. After exposure to Storage Phosphor Screen, radioactive labelled proteins were detected and quantified with the Typhoon PhosphorImager.

2.9 RNA immunoprecipitation, Slot-blot, and Mass spectrometry

For chloroplast RNA immunoprecipitation, 100 µl Sepharose-coupled protein A equilibrated with Co-IP Buffer (150 mM NaCl, 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$ and 0.5% (v/v) NP-40), were used to pre-incubate with 0.5 ml stroma (1 mg/ml) from different genotypes (the CSP41a-eGFP overexpressor, the CSP41b-eGFP overexpressor and cTP_{FNR}:eGFP) for 10 min to get rid off unspecifically bound proteins. After short time centrifugation, the supernatant was collected and incubated with 10 µl GFP antibody (Pierce) in a shaker for 2 hours at 4°C, followed by incubation with 100 µl Sepharose-coupled protein A (equilibrated with the Co-IP Buffer) for another 1 hour. The supernatant was kept for RNA extration, and the Sepharose was washed with 1 ml Co-IP Buffer 5 times. RNA was extracted by Phenol/Chloroform from the supernatant and the Sepharose pellet, and used either for chip analyses or Slot-blot as described (Schmitz-Linneweber *et al.*, 2005).

After RNA isolation, proteins were precipitated overnight from the Phenol/Chloroform phase by adding 5 volumes of Precipitation Solution (0.1 M NH_4OAc in 100% Methanol) at -20°C. After 20 min centrifugation at 4°C and 16000 *g*, the pellet was washed at least 4 times with Precipitation Solution and finally with 70% Ethanol. Precipitated proteins were solubilized in 2 times Laemmli Buffer (4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris-HCl pH 6.8), and separated on 15% SDS-PAGE. Samples were also analyzed with mass spectrometry.

For the PPP3 immunoprecipitation, thylakoids equivalent to 100 µg chlorophyll were washed 2 times with TMK Buffer and resuspended in 120 µl Thylakoid Co-IP Buffer (50 mM HEPES-KOH pH 8.0, 330 mM Sorbitol and 150 mM NaCl). Thylakoids were then solubilized by adding β-DM to the final concentration of 1%, and incubated with antibodies specific for GFP or PPP3. All pull-down procedures were performed as described in the RNA immunoprecipitation, except Thylakoid Co-IP Buffer containing 1% β-DM used in the washing step.

2.10 RNA stability assay

For radioactive labeling of *rrn23, psbCD* and *psbC* transcripts, the cDNA of respective was cloned into pGEM-T easy vector (Promega) under the control of the T7 promoter with primers (*rrn23*: 5'-TTCAAACGAGGAAAGGCTTACGG-3' and 5'-AGGAGAGCACTCATCTTGGG-3'; *psbCD*: 5'-ATAACATCAAAATCTATAA CATTAAGG-3' and 5'-GTTGGATGAATCTATTTTTCTC-3'; *psbC*: 5'-ATGAAAACCTTATATTCCCT GAG-3' and 5'-TTAGTTAAGAGGAGTCATGG-3'). *In vitro* transcription was performed in the presence of 12.5 µCi of [α-^{32}P] CTP in a final volume of 20 µl containing T7 RNA polymerase (Fermentas), according to the manufacturer's instructions. After 60 min incubation at 25°C, RNAs were precipitated and washed with Ethonal. 1/10th of the labeled transcripts was used in each assay. Labeled RNA was incubated with 1x10^7 chloroplasts in Chloroplast Lysis Buffer (50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$ and 10 mM β-mercaptoethanol) for 5 min at room temperature under a light intensity of 120 µmol m^{-2} s^{-1}. RNAs were recovered by Phenol/Chloroform extraction and separated by 1.5% denaturing agarose gel electrophoresis. Afterwards, RNAs were blotted to Hybond N^+ membrane and signals were detected according to Northern blot (Part 2.2).

2.11 Diagonal PAGE analyses

Diagonal PAGE analyses were performed as described (Ströher and Dietz, 2008), but with modifications. In brief, 50 µg stromal proteins were either reduced with 100 mM DTT or oxidized with 100 mM diamide at room temperature for 30 min, followed by incubation with 100 mM iodoacetamide in the dark for 30 min. Non-reducing SDS Laemmli Buffer was added, and the treated stroma was subject to 12% SDS-PAGE. After electrophoresis, the lanes contained the reduced or oxidized stromal proteins were cut off, and reduced in the Laemmli Buffer containing 100 mM DTT. Afterwards, the gel slices were applied to 12% SDS-PAGE for the second dimension, followed by Western blot analyses.

2.12 Iso-Electric Focusing

500 µg of stromal proteins were precipitated by 80% ice cold acetone. The pellet was dissolved in 350 µl Hydration Buffer (7 M Urea, 2 M Thiourea, 2% (w/v) CHAPs, 0.5% (v/v) Pharmalyte, 0.002% Bromophenol Blue, and 18.2 mM DTT) and resolved on Immobiline^{TM} Drystrip (gradient pH 3-10 NL, 18cm, GE healthcare). The isoelectric focusing program of Ettan IPGphor II was set as suggested by the manual (GE healthcare).

After Iso-Electric focusing, the gel strip was equilibrated in Equilibration Buffer 1 (6 M Urea, 75 mM Tris-HCl pH 8.8, 29.3% (v/v) Glycerol, 2% SDS, 0.002% Bromophenol Blue and 65 mM DTT) for 15 min, followed by incubation in Equilibration Buffer 2 (6 M Urea, 75 mM Tris-HCl pH 8.8, 29.3% (v/v) Glycerol, 2% SDS, 0.002% Bromophenol Blue and 135 mM Iodoacetamid) for another 15 min. Subsequently, the gel strip was resolved on Tricine-SDS-PAGE.

2.13 Bioinformatic sources

For gene model analysis and Blast search, TAIR (http://www.arabidopsis.org) and NCBI (http://www.ncbi.nlm.nih.gov/) were used. Sequences alignment and phylogenetic tree building were performed by Clustalw (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and Treeview (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) Protein domains were predicated online (http://www.ebi.ac.uk/Tools/pfa/iprscan/).

2.14 Primers list

Name	**Sequence 5'-3'**
csp41a C247S s	AACAACAAAGACTCCGAAGAATGGTTC
csp41a C300S s	AACATCTTCAACTCCGTTAGCGACAGA
csp41a C315S s	ATGGCCAAGCTCTCTGCTGCCGCTGCT
csp41a C247S as	GAACCATTCTTCGGAGTCTTTGTTGTT
csp41a C300S as	TCTGTCGCTAACGGAGTTGAAGATGTT
csp41a C315S as	AGCAGCGGCAGCAGAGAGCTTGGCCAT
csp41b C161S s	CAGTACATCTACTCTTCTTCAGCTGGT
csp41b C176S s	ATCTTGCCACATTCTGAGGAGGATGCA

csp41b C288S s	TTAGCAAAAGCTTCCGCAAAGGCCGGT
csp41b C161S as	ACCAGCTGAAGAAGAGTAGATGTACTG
csp41b C176S as	TGCATCCTCCTCAGAATGTGGCAAGAT
csp41b C288S as	ACCGGCCTTTGCGGAAGCTTTTGCTAA
csp41a pdnr s	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGCGGCTTTATCATCCTCCT
csp41a pdnr as	GGGGACCACTTTGTACAAGAAAGCTGGGTTAGCGGCCACTGGAGTTTTGAG
csp41b pdnr s	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGCGAAGA TGATGATG
csp41b pdnr as	GGGGACCACTTTGTACAAGAAAGCTGGGTTTTGAAGAACAAGTTTCTTGCT
slr1540-F	ATGCGCATTTTAATTATGGG
slr1540-R pdnr	GGGGACCACTTTGTACAAGAAAGCTGGGTTACTGAATGCCAGGATTTGCT
csp41b cTP-R	CCCATAATTAAAATGCGCATCTTTTCGCTCGACGCTGAAACATAG
ppp3-F pdnr	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGTCTTTAGCTCCGAGCAG
ppp3-R pdnr	GGGGACCACTTTGTACAAGAAAGCTGGGTTTTTCTTGTTAAAGCCAAACA
psaB-s	ATGGCATTAAGATTTCCAAGG
psaB-as	TTAACCGAATTTGCCCGATG
psaB-750s	GGAACTGCCATTCTAACCCTTC
psaB-750as	GAAGGGTTAGAATGGCAGTTCC
psaB-1532s	CAATAGGTCCTGGAGATTTCTTGG
psaB-1532as	CCAAGAAATCTCCAGGACCTATTG
psbC-s	ATGAAAACCTTATATTCCCTGAG
psbC-as	TTAGTTAAGAGGAGTCATGG
psbC-700s	TTGGAGGACATGTATGGTTAG
psbC-700as	CTAACCATACATGTCCTCCAA
Rrn23-s	TTCAAACGAGGAAAGGCTTACGG
Rrn23-as	AGGAGAGCACTCATCTTGGG
Rrn23-700s	TCTATGACCAGGATGAAGCT
Rrn23-700as	AGCTTCATCCTGGTCATAGA
Rrn23-1400s	GGACAACAGGTGAATATTCC
Rrn23-1400as	GGAATATTCACCTGTTGTCC
Rrn23-2100s	TGGGATTGGCTTTGGGCTTTT
Rrn23-2100as	AAAAGCCCAAAGCCAATCCCA
psbCD s	ATAACATCAAAATCTATAACATTAAGG
psbCD as	GTTGGATGAATCTATTTTTCTC
psaA s	GATTATTCGTTCGCCGGAAC
psaA as	TGGAGCTGCTTTGTGATAATG
rbcL s	CGTTGGAGAGACCGTTTCTT
rbcL as	CAAAGCCCAAAGTTGACTCC
rrn16 s	GCTTAACACATGCAAGTCGG
rrn16 as	AAAAAGCCACCTACAGACGC
atpB s	TGAGAACAAATCCTACTACTTC

atpB as	TTTTCCCCCACGACGATAAG
rps18 s	GAATAAATCTAAGCGACTTTTTAC
rps18 as	ATTTTTTTCTGGTTCTAAGACTAG
psbA s	TGCATCCGTTGATGAATGGC
psbA as	CTCACGGTTATTTTGGCCGA
Ds3´-1	CGATTACCGTATTTATCCCGTTCG
Fish1	CTGGGAATGGCGAAATCAAGGCATC
PPP3-1s	ATGTCTTTAG CTCCGAGCAG
PPP3-371as	TATCGCCAACAGTGAACTTTC
PPP3-951s	CGTGAAGGGC TGCAATTTTC
PPP3-1800as	AGACACAAACTTTAGGTGAAC

Table 2.1 Oligonucleotides employed in Materials and methods

3 Results

The Putative Photosynthetic Protein 3

3.1 PPP3 is a chloroplast protein existing in eukaryotic photoautotroph species

PPP3 is 335 amino acids long, and is encoded by the *Arabidopsis* nuclear genome. According to ChloroP prediction (Emanuelsson *et al*., 1999), PPP3 comprises a 73 amino-acid long chloroplast transit peptide at the N-terminal. Analysis with InterProScan (Zdobnov and Apweiler, 2001) reveals that PPP3 contains a PDZ-like domain (58-137 amino acids) and a TPR-like domain (222-317 amino acids) (Figure 3.1 B).

By BLAST searches against the non-redundant protein sequence database in NCBI, homologs of PPP3 were found in almost all eukaryotic photosynthetic organisms, but neither in red algae nor in cyanobacteria. PPP3 homologs in Arabidopsis (*Arabidopsis thaliana*), soybean (*Glycine max*), poplar (*Populus trichocarpa*), rice (*Oryza sativa*), moss (*Physcomitrella patens*) and Chlamydomonas (*Chlamydomonas reinhardtii*) were aligned, and a phylogenetic tree was built by using ClustalW and Treeview (Figure 3.1 A). This tree suggests that all PPP3 homologs in the eukaryotic photoautotroph species evolved from the same ancestor.

3.2 PPP3 knock-out mutants and PPP3-eGFP overexpressors show the same phenotype as the wild type

To characterize the biological function of PPP3, transposon and T-DNA insertion knock-out mutants in *Arabidopsis* were employed. Three independent *ppp3* mutant lines were screened for homozygous plants. The exact insertion points were confirmed by sequencing the amplified PCR product and are shown in the *PPP3* gene model in Figure 3.1 C. For *ppp3-1* and *ppp3-3,* the *DS* elements were inserted in the 1st and 5th exon, respectively. *ppp3-2* is a T-DNA insertional mutant leading to a disruption in the 1st exon.

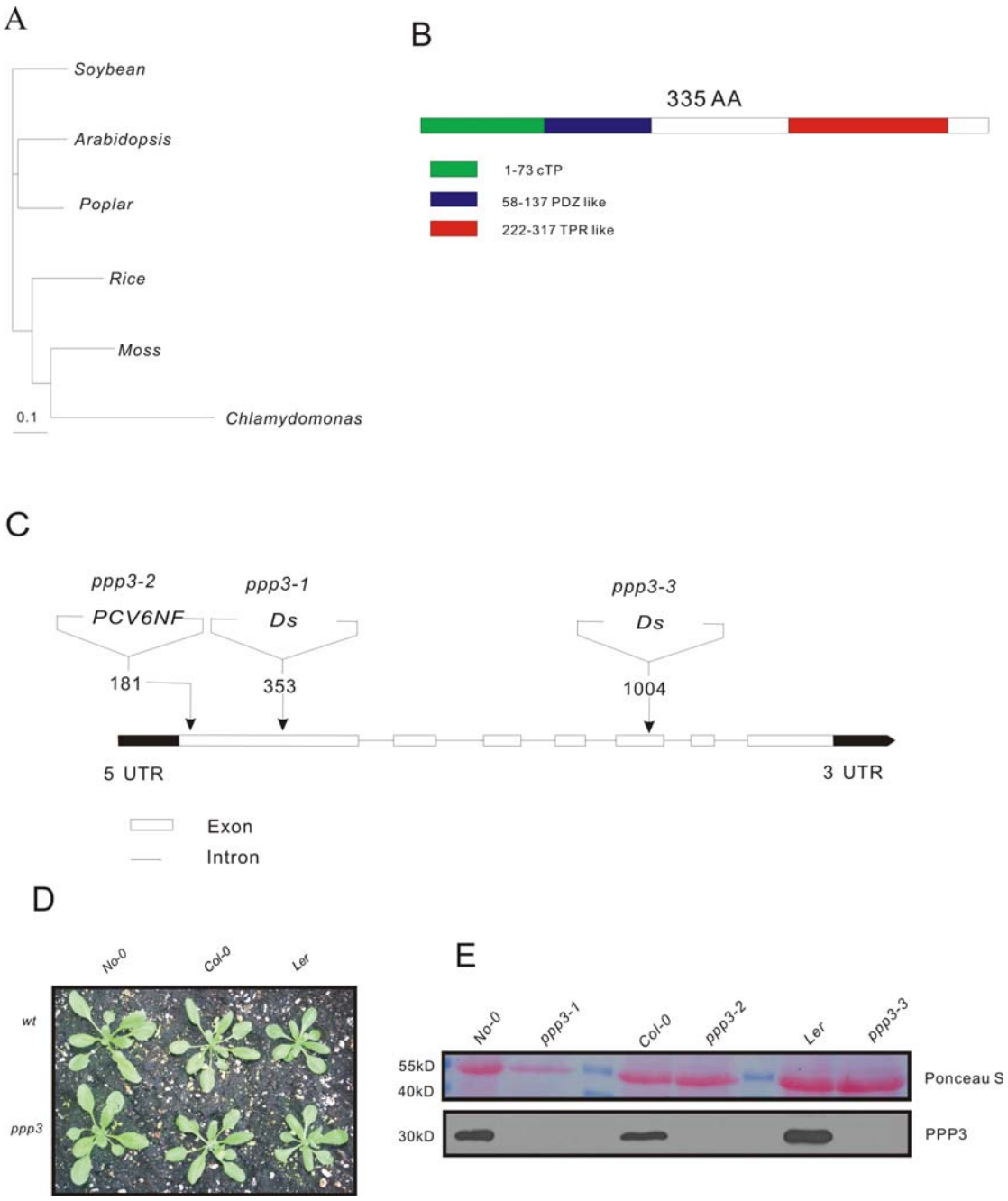


Figure 3.1 Phylogenetic tree and protein domain prediction of PPP3; gene model and phenotype of Transposon and T-DNA insertion mutant lines

(A) By Blast searches, PPP3 homologs in Arabidopsis (GI: 18405391), soybean (GI: 255639295), poplar (GI: 224073126), rice (GI: 50509325), moss (GI: 168006574) and Chlamydomonas (GI: 159463656) were obtained from NCBI, and the phylogenetic tree was built by using ClustalW and Treeview.

(B) Protein domains were predicted by ChloroP and InterProScan. The cTP is shown by a green box, the PDZ-like domain by a blue box, and the TPR-like domain by a red box.

(C) T-DNA or transposon insertion positions of three *ppp3* mutant lines are shown on the gene model of *PPP3* (TAIR) in Arabidopsis. Exons are shown by open boxes, introns by conjunctive black lines,

and untranslated regions (UTR) by black boxes. The insertion sites and the inserted elements are provided for each mutant line.
(D) Growth phenotype of the three independent *ppp3* insertion mutant lines and the corresponding wild types. Plants were grown in the green house under long day condition for 3 weeks.
(E) Western blot analyses with PPP3-specific antibodies raised against total protein extract (20 µg) from *ppp3* mutants and wild types. RBCL detected by Ponceau S staining was used as a loading control.

By Western blot analyses with the PPP3-specific antibody raised against total proteinextract from mutants and wild type lines, it could be shown that all *ppp3* mutants are complete knock-out lines (Figure 3.1 E). The molecular mass of PPP3 detected only in the wild type, is around 30 kDa, which corresponds to the predicted size of PPP3 without the cTP. However, all homozygous *PPP3* knock-out lines, grown either on soil (green house or climate chamber) (Figure 3.1 D) or on MS plates (Salt stress and drought stress), show no significant altered phenotype compared to the wild types. Several PPP3-eGFP overexpressors in the *ppp3* mutant and Col-0 background also show the same growth phenotype as the wild type.

To investigate if PPP3 is involved in photosynthesis, double mutants were obtained by crossing the *ppp3-2* mutant with different photosynthetic mutants (*ppp3*psad1* (PSI deficient mutant, Ihnatowicz *et al.*, 2004), *ppp3*pam68* (PSII assembly mutant, Armbruster *et al.*, 2010), *ppp3*pete2* (Plastocyanin mutant, Pesaresi *et al.*, 2009), *ppp3*pgr5* (Cyclic electron flow deficient mutant, Munekage *et al.*, 2002), *ppp3*prpl11* (Chloroplast translation deficient mutant, Pesaresi *et al.*, 2001) and *ppp3*csp41b* (Chloroplast RNA metabolism, Bollenbach *et al.*, 2009)), and these double mutants showed no additional phenotype.

3.3 Subcellular localization of PPP3

After chloroplast fractionation, proteins from intact chloroplasts, the stroma and thylakoids were employed for Western blot analysis. The result that PPP3 was only detected in thylakoids (Figure 3.2 A), is consistent with an earlier proteomic study, in which PPP3 was found to be associated with the thylakoid membrane of Arabidopsis (Peltier *et al.*, 2004).

To study if PPP3 is either localized in the stroma lamellae or in the grana thylakoids were fractionated and antibodies againstsubunits of different photosynthetic complexes were used as controls (Figure 3.2 B). For instance, PSI subunits (PsaC and PsaD) and the $Cytb_6f$ subunit PetC were enriched in the stroma lamellae; the PSII subunit PsbD was enriched in the grana; PPP3 was mainly enriched in the stroma lamellae and in the final supernatant fraction (s.n.). Taken those results together, PPP3 is localized in the stroma lamellae like the PSI complex and the Cytb6f complex. However, the abundant signal of PPP3 in the supernatant fraction implies that PPP3 might be loosely attached to the stroma lamellae.

PPP3 is associated to the thylakoid membrane (Figure 3.2 A), but no transmembrane domain is predicted by InterProScan (Figure 3.1 B). Treatments with chaotropic salts or alkaline solutions were performed to analyze how PPP3 is anchored to the thylakoid membrane.Chaotropic salts (2M) are used to break electrostatic interactions and a solution with an alkaline pH to destroy hydrophobic interactions (Karnauchov *et al.*, 1997). In comparison to Lhcb that can resist to all salt treatments, PPP3 were washed out both by chaotropic salts and alkaline solutions (Figure 3.2 C). Additionally, PPP3 was detected in the supernatant during the thylakoid fractionation (Figure 3.2 B). These results imply that PPP3 is attached to the thylakoid lamellae by hydrophobic and by electrostatic interactions. Since the fact that alkaline solutions washed out almost all PPP3 from the thylakoid membrane, it can be deduced that PPP3 is attached to the thylakoid membrane mainly by hydrophobic interactions, but only weak electrostatic interactions.

3.4 Photosynthetic Complexes are not affected in the *ppp3* mutant

Since *PPP3* is co-regulated with photosynthetic genes in regulon 1 (Biehl *et al.*, 2005), BN-PAGE with the wild type and the *ppp3* mutant were performed to examine whether PPP3 is a part of one of the photosynthetic complexes. Thylakoid membranes from the *ppp3-1* and *No-0* were solubilized with either digitonin or β-DM, and photosynthetic complexes were separated by Blue Native PAGE (Figure 3.3 A). The 2^{nd} dimension of Blue Native PAGE was either blotted for immunodetection assays or used for silver staining. As shown by immunodetection, PPP3 can form a small complex around 60 kDa (hetero-dimer or homo-dimer of PPP3) on the thylakoid

membrane (Figure 3.3 B). Compared to PetC (Rieske) from the $Cytb_6f$ complex, PPP3 is not a component of any photosynthetic complexes. Silver staining shows that there is no change in the composition of photosynthetic subunits (Figure 3.3 C).

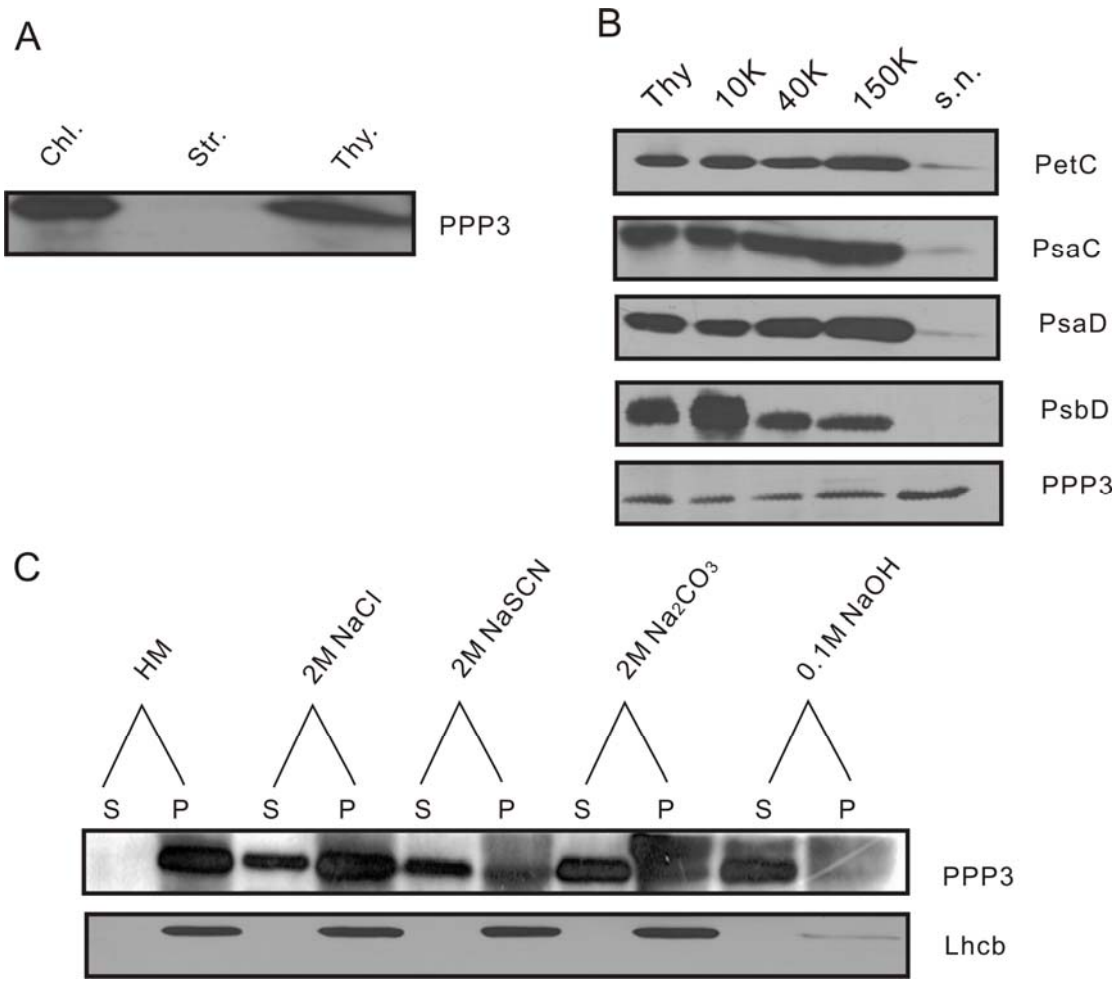


Figure 3.2 Subcellular localization of PPP3

(A) Western blot analysis with the PPP3 antibody which was raised against stromal proteins (Str), thylakoid proteins (Thy) and proteins of intact chloroplasts (Chl). Thylakoid and chloroplast containing 10 μg of chlorophyll were used, and stromal proteins were extracted from intact chloroplasts having the same amount of chlorophyll.

(B) Thylakoid membranes treated shortly with digitonin were fractionated by differential centrifugation. The 10K pellet represents thylakoid grana, the 40K pellet intermediate membranes, and the 150K pellet stroma lamellae. Antibodies raised against different subunits of the photosynthetic complexes (PsaC and PsaD for PSI, PetC for cytb6f, and PsbD for PSII) were used as controls.

(C) Thylakoid membrane salt treatments. Thylakoids were washed with 2M NaCl, 2M NaSCN, 0.1M Na_2CO_3, and 0.1 M NaOH in HM buffer. Pellets (P) and supernatants (S) of each treatment were collected for Western blot analysis with the PPP3 antibody and the Lhcb antibody.

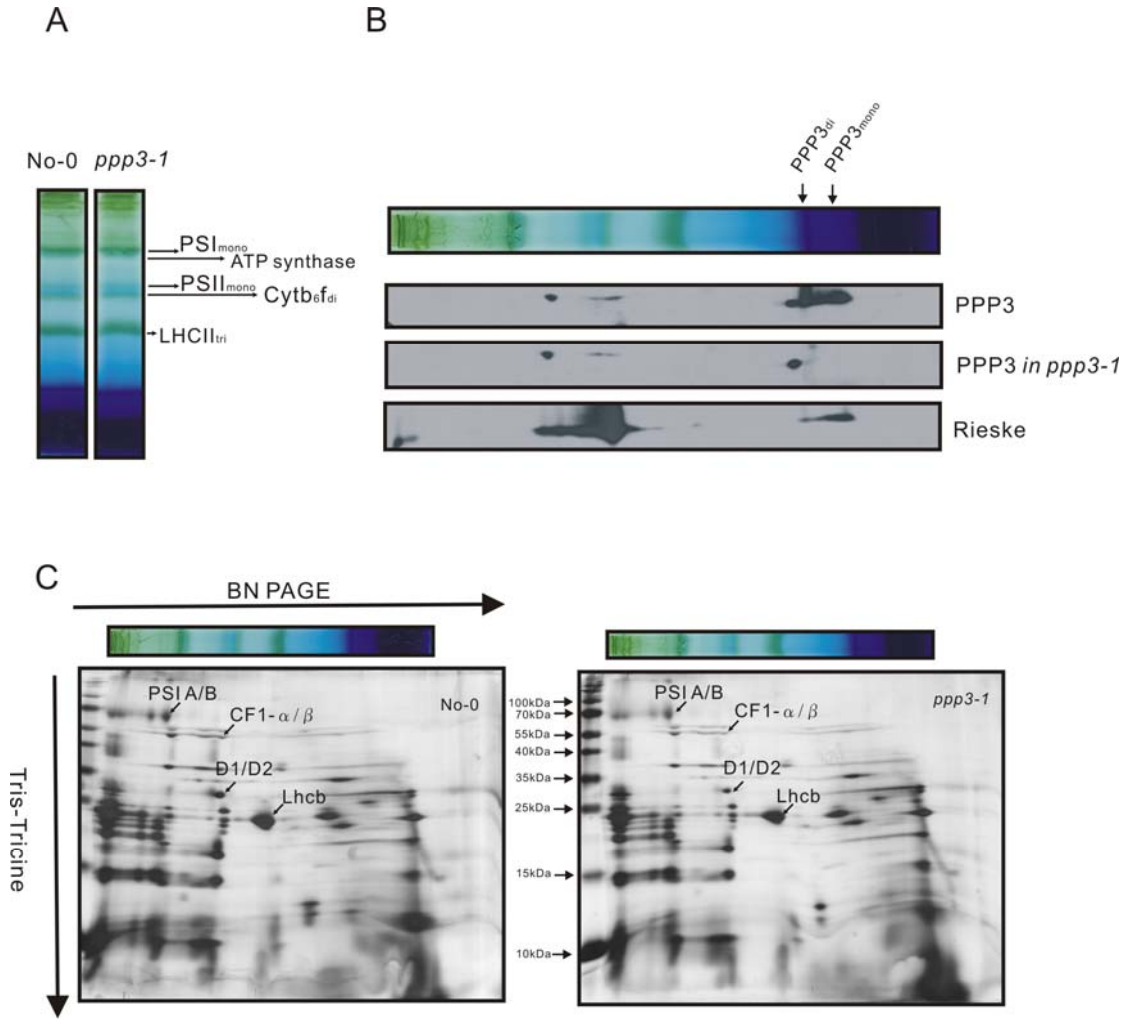


Figure 3.3 BN-PAGE analyses to the *ppp3* mutant and the wild type

Thylakoids from both *ppp3-1* and *No-0* solubilized by digitonin were seperated on 4%-18% BN-PAGE gel. Gel slices from the first dimension were denatured and subunits of complexes were separated on Tricine-SDS-PAGE gel for the 2nd dimension. The 2nd dimension gels were either blotted on PVDF membranes for western analyses or were silver stained.

(A) The first dimension of BN-PAGE with thylakoids from *ppp3-1* and *No-0*, showing the different photosynthetic complexes.

(B) Western blot analyses with PPP3 and Rieske (PetC) antibodies. PPP3 dimer and monomer were marked with vertical arrows while the *ppp3-1* mutant was used as a negative control.

(C) Silver staining of the 2nd dimension from *ppp3-1* and *No-0* show that there is no change on the distribution and abundance of thylakoid membrane proteins.

The Putative Photosynthetic Protein 1

3.5 The *csp41b* mutant has a light dependent phenotype.

The phenotype of *csp41b* was reported to show pale green leaves, growth retardation both on MS plates containing 1% sucrose (Hassidim *et al.*, 2007) and on soil (Bollenbach *et al.*, 2009). Here, the same pale green phenotype of *csp41b* was observed (Figure 3.4 A), and the growth retardation phenotype was getting severe with increasing light intensities. When the *csp41b* mutant was grown under low light intensity (80μmol $m^{-2}s^{-1}$) in the climate chamber (12h dark/12h light), it had the same leaf size as the wild type, but leaves of *csp41b* mutant were still pale green. When the *csp41b* mutant was grown under moderate light intensity (120μmol $m^{-2}s^{-1}$) in the climate chamber (12h dark/12h light), it was smaller than Col-0 grown under the same condition.Nonetheless, the *csp41b* mutant grown under 120μmol $m^{-2}s^{-1}$ shows the same size as grown under 80μmol $m^{-2}s^{-1}$. The *csp41b* mutant grown in the green house, where the light intensity is usually above 180μmol $m^{-2}s^{-1}$, shows the most severe phenotype with respect to the wild type. With increasing light intensities, the leaf size of the *csp41b* mutant was reduced, but that of the wild type was increased. These results indicate that the *csp41b* mutant can not cope with higher light intensity as the wild type.

3.6 CSP41a-eGFP overexpressors show a growth retardation phenotype, but the CSP41b-eGFP overexpressor does not

Three independent CSP41a-eGFP overexpressors (*CSP41a-eGFP OE1, 2 and 3*) in the *csp41a* background and one CSP41b-eGFP overexpressor (*CSP41b-eGFP OE*) in the Col-0 background were generated (Figure 3.4 D, E & F). Two independent *csp41a* mutants (61.668 and 91.154) screened from the T-DNA mutant collection of C. Koncz show the same phenotype as the wild type, which is consistent with previous published results (Beligni and Mayfield, 2008). Despite no obvious phenotype in *csp41a* mutants, three independent CSP41a-eGFP overexpressors in the *csp41a* background (61.668) are characterized by a slower growth rate under green house condition (Figure 3.4 B). The leaf areas of *CSP41-eGFP OE1* and *CSP41-eGFP OE2*

are reduced to about 25% compared to that of the wild type, and the leaf area of *CSP41e-GFP OE2* is reduced to 50%. Instead, *csp41a* mutants grow as fast as Col-0 (Figure 3.4 C). The *CSP41b-eGFP OE* line in the *Col-0* background has the same phenotype like Col-0.

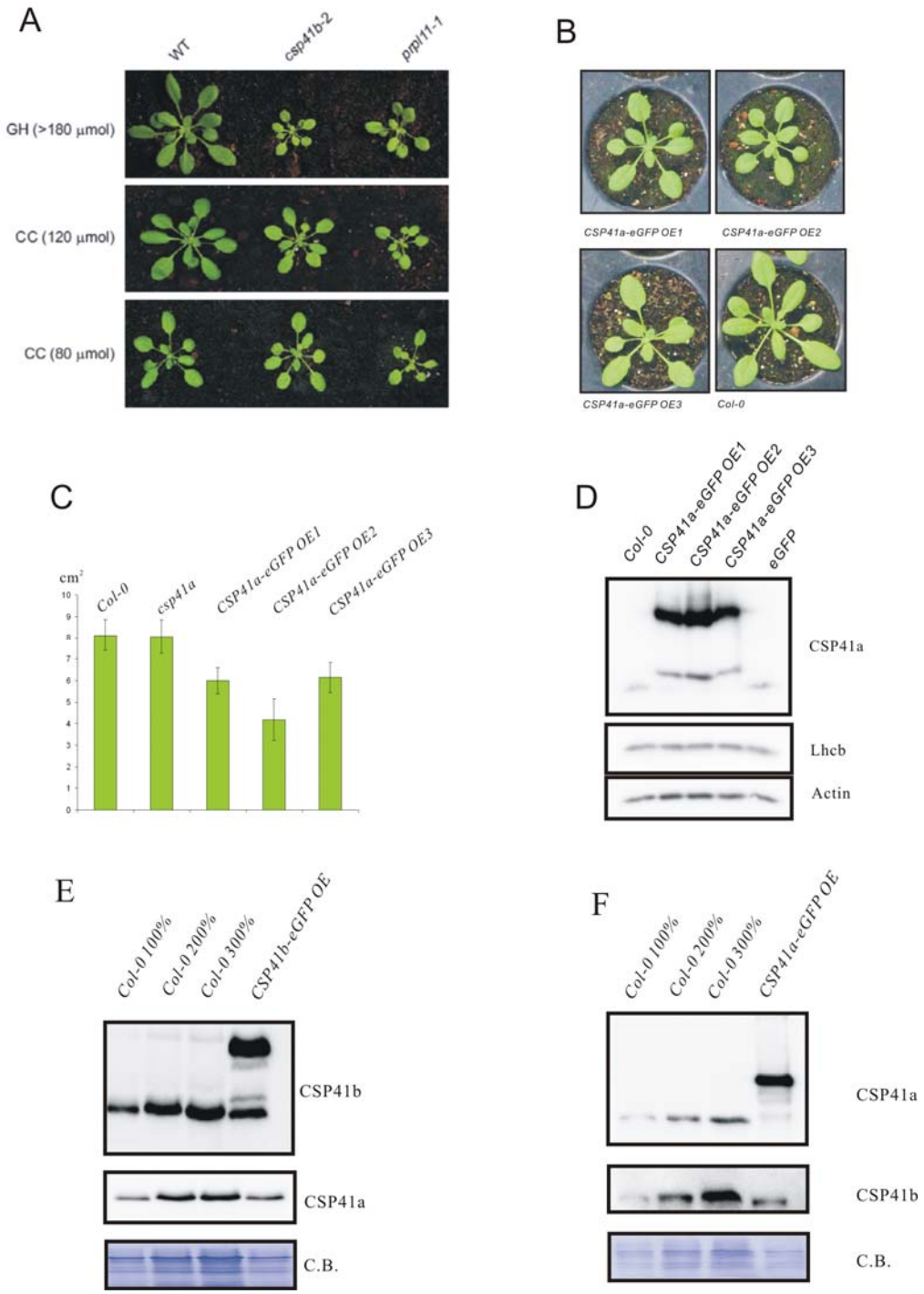
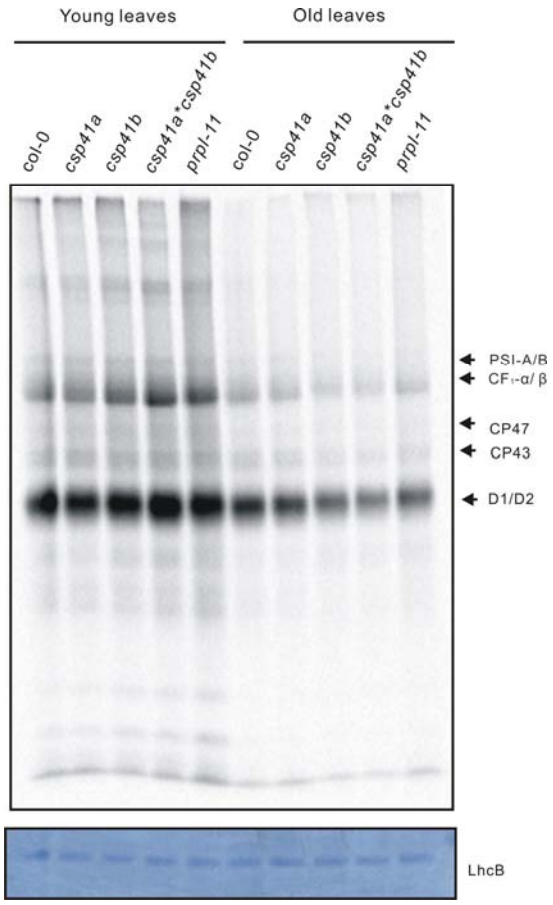


Figure 3.4 Phenotype of the *csp41b* mutant and CSP41a-eGFP overexpressors, and protein levels in these overexpressors

(A) The *csp41b* mutant shows a light-dependent phenotype. Col-0, *csp41b* mutants, and *prpl11* mutants were grown under different light intensities ranging from 80µmol $m^{-2}s^{-1}$ (climate chamber, CC) to more than 180µmol $m^{-2}s^{-1}$ (green house, GH). Pictures were taken after 3 weeks.
(B) Pictures of *Col-0* and CSP41a-eGFP overexpressors (3 weeks old) under green house condition.
(C) The leaf area was calculated using ImageJ to show the growth retardation phenotype (3 weeks old plants) of three different CSP41a-eGFP overexpressors.
(D) Overexpression levels of CSP41a-eGFP in CSP41a-eGFP overexpressors. 5 µg of total protein from leaves were seperated on 12% SDS-PAGE gel. The CSP41a was immunodetected to determine CSP41a-eGFP levels. Signals were adjusted to Lhcb and Actin amounts.
(E & F) The amount of CSP41a in the CSP41b-eGFP overexpressor and the amount of CSP41b in the CSP41a-eGFP overexpressor are shown. 5, 10, and 15 µg of total protein from wild type, and 5 µg of total protein from the overexpressor were separated by 12% SDS-PAGE and subsequently transferred to PVDF. CSP41 protein levels were detected with specific antibodies. The PVDF membrane stained by Coomassie Blue R 250 was taken as the loading control.

3.7 The CSP41b and CSP41a level increases in the CSP41a-eGFP overexpressor and in the CSP41b-eGFP overexpressor, respectively

CSP41a and CSP41b can form complexes *in vivo* and the CSP41a level is dependent on CSP41b (Peltier *et al.*, 2006; Bollenbach *et al.*, 2009). On the basis of those results, it is deduced that the accumulation of CSP41a can influence the level of CSP41b, and *vice versa*. Therefore, the level of CSP41b (in the CSP41a-eGFP overexpressor) and CSP41a (in the CSP41b-eGFP overexpressor) were determined by Western blot analyses. In *CSP41a-eGFP OE1* (CSP41a-eGFP accumulated more than 10 times compared to CSP41a in Col-0), the accumulation of CSP41b increases up to 150% compared to that in Col-0 (Figure 3.4 F). In *CSP41b-eGFP OE* (CSP41b-eGFP accumulated more than 6 times compared to CSP41b in Col-0), the level of CSP41a also increases up to 150% compared to that in Col-0 (Figure 3.4 E). Due to CSP41b-eGFP overexpression in the Col-0 background, the increase of the wild type form of CSP41b could also be observed, which means that CSP41b can form complexes with CSP41a, but also with itself. The latter case is consistent with that the CSP41b complexes still can be detected by BN-PAGE in the *csp41a* mutant described by Armbruster (unpublished data).

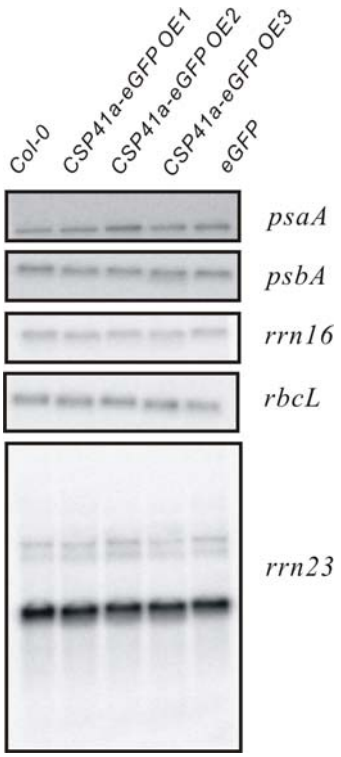


4 weeks old plants (Green house) with 12 rosette-leaf stage were used for *in vivo* labeling studies. Young (8th true leaf) and old leaves (4th true leaf) from Col-0, *csp41a, csp41b, csp41a*csp41b* and *prpl11* were labeled with ^{35}S-Methionine for 30 min. Cycloheximid was used to inhibit cytosolic translation. Thylakoids extract from labeled leaves were normalized to the amounts of LhcB, and separated on Tricine-SDS-PAGE. Accumulations of several subunits (D1, D2, CP43, CP47, PSI-A/B, and CF_1-α/β) from photosynthetic complexes are highlighted by arrows.

Figure 3.5 *In vivo* labeling of Col-0*, csp41a, csp41b, csp41a*csp41b* and *prpl11*

3.9 The transcript abundance in *CSP41a-eGFP OE* lines is not affected

The RNA endoribonuclease activity of CSP41a was reported *in vitro* using heterologously expressed protein (Yang *et al.*, 1996). The degradation rate of *psbA* and *rbcL* transcripts decreased in CSP41a knock-down lines in tobacco (Bollenbach *et al.*, 2003). To check if CSP41a exhibits an endoribonuclease activity *in vivo*, and if the growth retardation phenotype of *CSP41a-eGFP OE* lines is caused by an increased RNA turnover rate, the transcript abundance of *psaA*, *psbA*, *rbcL*, *rrn16* and *rrn23* was examined by Northern blots (Figure 3.6). However, accumulations of these transcripts were not affected, which suggests that CSP41a is not an endoribonuclease.



Total RNA was extracted from 3-week-old plants (Col-0, *CSP41a-eGFP OE* lines and *eGFP* line) grown in the green house. 5µg of total RNA of each line were separated on 1.5% denaturing agarose gel and transferred to Hybond N^+ membranes. Probes of *psaA, psbA, rrn16, rbcL* and *rrn23* generated by PCR and labeled with ^{32}P-dCTP were hybridized for the determination of transcript levels.

Figure 3.6 Northern blots with total RNA from *CSP41a-eGFP OE* lines

3.10 CSP41a and CSP41b are RNA-binding proteins *in vivo*.

It was previously shown that CSP41a binds to the stem loop structure at 3' UTR of *petD* mRNA *in vitro* (Chen *et al.*, 1995). To examine if CSP41a and CSP41b have a RNA-binding ability *in vivo*, the stroma isolated from Col-0 was treated with heparin, RNase inhibitor and RNase A before separation on the BN-PAGE gel. Heparin is a helical polysaccharide (Capila and Linhardt, 2002), which can dissociate RNA-Protein complexes (Tanguay and Gallie, 1996). In addition, RNase inhibitor or RNase were used to protect RNA or to degrade RNA. In the non-treated stroma (Figure 3.7 A & B), CSP41a and CSP41b could be detected from around 40 kDa (monomer) to above 1MDa, which was shown in earlier studies (Yamaguchi *et al.*, 2003; Peltier *et al.*, 2006; Olinares *et al.*, 2010; Schroter *et al.*, 2010). After heparin treatment, high molecular complexes (above 500 kDa) of CSP41a and CSP41b disappeared. The same phenomenon was also observed in the RNase-treated stroma, but not in the RNase inhibitor-treated stroma. Taken those results together, higher molecular complexes of CSP41a and CSP41b are assumed as RNA-CSP41 complexes, and CSP41a and CSP41b are RNA-binding proteins.

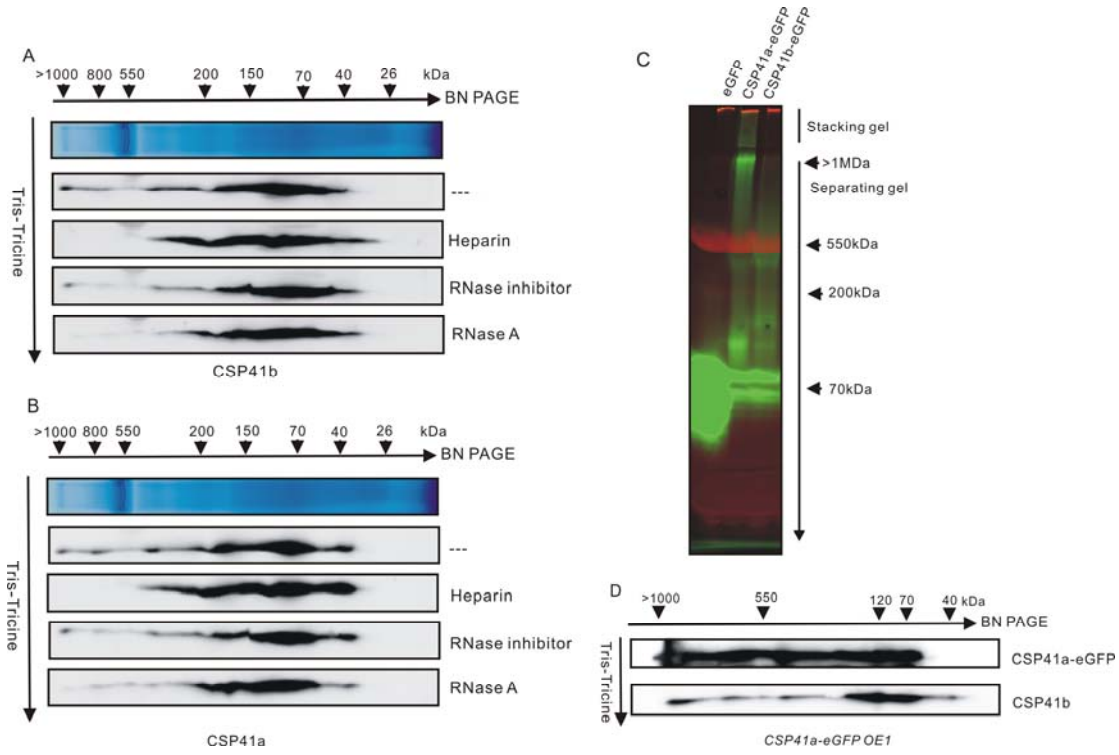


Figure 3. 7 CSP41a and CSP41b are RNA-binding proteins

(A & B) Complexes associated with CSP41 from 40 kDa to above 1 MDa distributed on 2D BN-PAGE gels were detected with CSP41 specific antibodies. The stroma of Col-0 was treated with heparin, RNase inhibitor and RNase A before samples were loaded on a 4-18% BN-PAGE. Dashed lines (---) indicate no treatment.

(C) Fluorescence scanning of the 1st D BN-PAGE stroma separation of eGFP, *CSP41a-eGFP OE* lines and the *CSP41b-eGFP OE*. The picture was merged from a eGFP fluorescence scan (Green, excited by the 488 nm blue laser) and a protein fluorescence scan (Red, excited by the 633 nm laser).

(D) The stroma of *CSP41a-eGFP OE* lines was separated on the 2D BN-PAGE gel and detected with CSP41a- and CSP41b-specific antibodies.

Stroma from *CSP41a-eGFP OE1* and *CSP41b-eGFP OE* lines was separated on a BN-PAGE gel, and scanned by applying different fluorescence filters. Pictures with different fluorescence signals from two scans were merged (Figure 3.7 C). The fluorescence signal (Red) at 550 kDa emitted by RuBisCo complexes was excited by a 633 nm laser, and the fluorescence signal (Green) emitted by eGFP was excited by a 488 nm laser. The distribution of fluorescence signals of eGFP shows the same pattern as the signal distribution detected by immunodetection analysis with CSP41 antibodies. Stroma from the eGFP line was used as a negative control to show that eGFP is not bound to RNA. In addition, more CSP41a-eGFP was accumulated at

1MDa and above 1Mda (in the 4% stacking gel) than in the CSP41b-eGFP sample. This implies that CSP41a and CSP41b can form different complexes in the CSP41a-eGFP and the CSP41b-eGFP overexpressors.

Stroma from the *CSP41a-eGFP OE1* line was also analyzed by 2D BN-PAGE (Figure 3.7 D). It is shown that in the *CSP41a-eGFP OE1* line, CSP41a-RNA complexes above 200 kDa accumulated more than CSP41b-RNA complexes. These results imply that the presence of more CSP41a-RNA complexes could be caused by its highly overexpressed level.

3.11 RNA targets of CSP41 proteins and CSP41b can stabilize targets RNA

To find RNA targets of CSP41a and CSP41b *in vivo*, RNA immunoprecipitation (RIP) was carried out by incubating GFP antibodies with the stroma of *CSP41a-eGFP OE1*, *CSP41b-eGFP OE* and eGFP lines. After incubation, RNA was extracted from the supernatant and the pellet fraction, and subjected either to RIP-Chip or to Slot-blot analysis. Proteins in the pellet were extracted from the phenol phase after RNA extraction, and identified by mass spectrometry analyses. Western blot analysis using GFP antibodies showed that the same amount of CSP41a-eGFP and CSP41b-eGFP was pulled down (Figure 3.8 C). Slot-blot analysis (Figure 3.8 A) using several probes (*psaA, psbA, rps18, atpB, rbcL, rrn16, rrn23* & *psbC*), showed that these transcripts were enriched to some extent in the pellet fraction of CSP41a-eGFP and CSP41b-eGFP. Since eGFP can not bind to RNA, stroma from eGFP lines was taken as a negative control.

The enrichment ratio of the negative control eGFP to each transcript ranges from 0.02 to 0.05. Instead, for CSP41a-eGFP and CSP41b-eGFP, the enrichment ratio for each transcript ranges from 0.53 to 9.63. The affinity of CSP41 for transcripts were defined by the enrichment ratio, and grouped into 3 categories: (i), low affinity (enrichment ratio below 1); (ii), moderate affinity (enrichment ratio 1-2) and (iii), high affinity (enrichment ratio above 2). Here, CSP41 has high affinity for *psaA, rbcL, atpB, psbC, psbA* and *rps18* transcripts (Figure 3.8 B), which are mRNAs for photosynthetic subunits and ribosomal proteins. However, both CSP41a and CSP41b have a low affinity for ribosomal RNAs, such as *rrn23* and *rrn16*

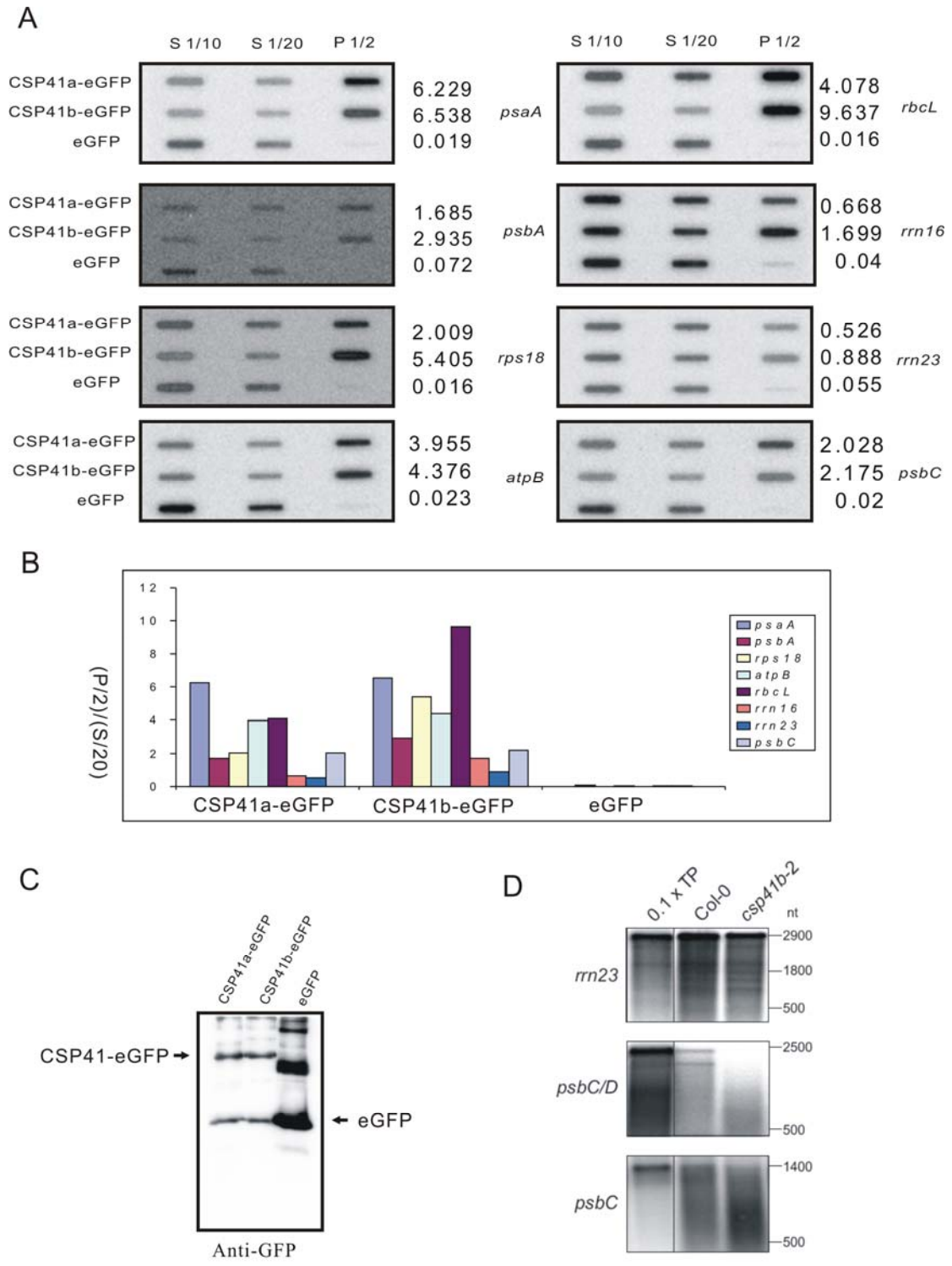


Figure 3.8 Chloroplast transcript targets of CSP41a and CSP41b

(A) Isolated stroma (0.5 mg) from *eGFP*, *CSP41a-eGFP OE1* and *CSP41b-eGFP OE* was incubated with the GFP antibody. RNA in the pellet and supernatant fraction were extracted with phenol/chloroform. Half of the RNA amount from the pellet (P 1/2) and two different dilutions of RNA from the supernatant (S 1/10 and S 1/20) were slot-blotted. Subsequently, transcritps were detected with several ^{32}P-dCTP labelled probes (*psaA, psbA, rps18, atpB, rbcL, rrn16, rrn23 & psbC*). The

enrichment ratio of CSP41a, CSP41b and eGFP of different transcripts was calculated on the basis of the signal intensity P 1/2 to that of S 1/20.

(B) The diagram shows the enrichment ratio of CSP41a, CSP41b and eGFP of different targets.

(C) Western blot analyses using a GFP antibody show that equal amounts of CSP41a-eGFP and CSP41b-eGFP were pulled down.

(D) ^{32}P-CTP labeled transcripts of *rrn23*, *psbC/D* and *psbC* were incubated with hypotonic broken chloroplasts from Col-0 and from *csp41b* mutants for 5 min. Labeled transcripts were re-isolated and separated on a denaturing agarose gel (1.5%), followed by transfer to Hybond N^+ membrane and radioautography.

It is obvious that CSP41 can pull down RNAs from the chloroplast stroma, but not all transcripts. Addtionally, the abundances of transcripts are different and some of the pulled-down CSP41 binds no RNA, hence, it is difficult to calculate the percentage of CSP41 proteins bound to a certain transcript. However, the percentage of a specific transcript bound by the CSP41 proteins relative to its total amount can be estimated (Table 3.1). For instance, it is calculated that around 50% of total *rbcL* transcript could be pulled-down by CSP41b-eGFP, which means that at least half of *rbcL* RNA interacts with CSP41b-eGFP in the stroma of CSP41b-eGFP overexpressors. Both CSP41a and CSP41b can interact with about 40% of the *psaA* transcript. Considering that the mature form of *psaA* and *rbcL* are the most abundant, after processing to their primary transcripts (Figure 3.9), we can conclude that CSP41a and CSP41b mainly bind to mature mRNAs *in vivo*.

P/(P+S)	*psaA*	*psbA*	*rps18*	*rbcl*	*atpB*	*psbC*	*rrn16*	*rrn23*
CSP41a-eGFP	>38.4%	>14.4%	>16.7%	>29.0%	>28.3%	>16.9%	>6.3%	>5.0%
CSP41b-eGFP	>39.5%	>22.7%	>35.1%	>49.0%	>30.4%	>17.9%	>14.5%	>8.2%
eGFP	0.1%	0.7%	0.1%	0.1%	0.2%	0.2%	0.4%	0.5%

Table 3.1 Ratios of the amount of CSP41 bound transcripts to that of total transcripts

The data from this table is calculated on the basis of the enrichment ratio from Figure 3.8 A. The enrichment ratio is assumed as R = (P/2)/(S/20), then P/S = R/10, and finally P/(P+S) = 1/(1+10/R). P is the transcript abundance in the pellet of RIP, and S is the transcripts abundance remained in supernatant. (P+S) is the total amount of one certain transcript.

Three target transcripts were chosen for the RNA stability assay, which were two monocistronic transcripts (*rrn23* and *psbC*) and one polycistronic transcript (*psbC/D*). These transcripts were generated with T7 RNA polymerase in the presence of ^{32}P-CTP, and incubated with hypotonic broken chloroplasts of Col-0 and *csp41b* mutants. Broken chloroplasts without CSP41b (the *csp41b* mutant) can degrade transcripts faster than broken chloroplasts with CSP41b (Col-0). These results suggest that CSP41b can stabilize its target transcripts.

3.12 CSP41-RNA complexes are regulated by the stromal redox state

Dark-adapted plants had more CSP41b-RNA complexes than light-adapted plants (Figure 3.9 A). CSP41b-RNA complexes accumulated more in *psad1* compared to Col-0 (Figure 3.9 B). The double mutant of *csp41b*psad1* has a pronounced growth retardation phenotype compared either to the *csp41b* mutant or to the *psad1* mutant (Figure 3.9 C). When the same amount of stroma from *psad1* and Col-0 were separated on the non-reducing SDS-PAGE gel, a dimer and a trimer of CSP41b could be detected at 76 kDa and 120 kDa, and the CSP41b dimer accumulated to a large extent in the *psad1* mutant (Figure 3.9 D). In the dark, the stroma is supposed to be oxidized because no reducing ferredoxin is generated by LEF. The stroma in *psad1* mutant, in which the PSI levels decrease to 50%, is assumed to be oxidized due to deficiency in LEF and accumulation of reactive oxygen species (ROS) (Ihnatowicz *et al.*, 2004). The fact, that CSP41b-RNA complexes are more accumulated in the oxidized stroma suggests that they could be regulated by the stromal redox state.

3.13 Cysteine-Serine mutants of CSP41b can complement the *csp41b* phenotype

By 2D reduction-oxidation diagonal SDS-PAGE and subsequent protein identification by MS analysis, CSP41a and CSP41b were found to form an intra-molecular disulphide bond and inter-molecular disulphide bonds, respectively (Ströher and Dietz, 2008). Those results were confirmed by Diagonal SDS-PAGE followed by Western blot analysis using CSP41a and CSP41b specific antibodies (Figure 3.10 A).

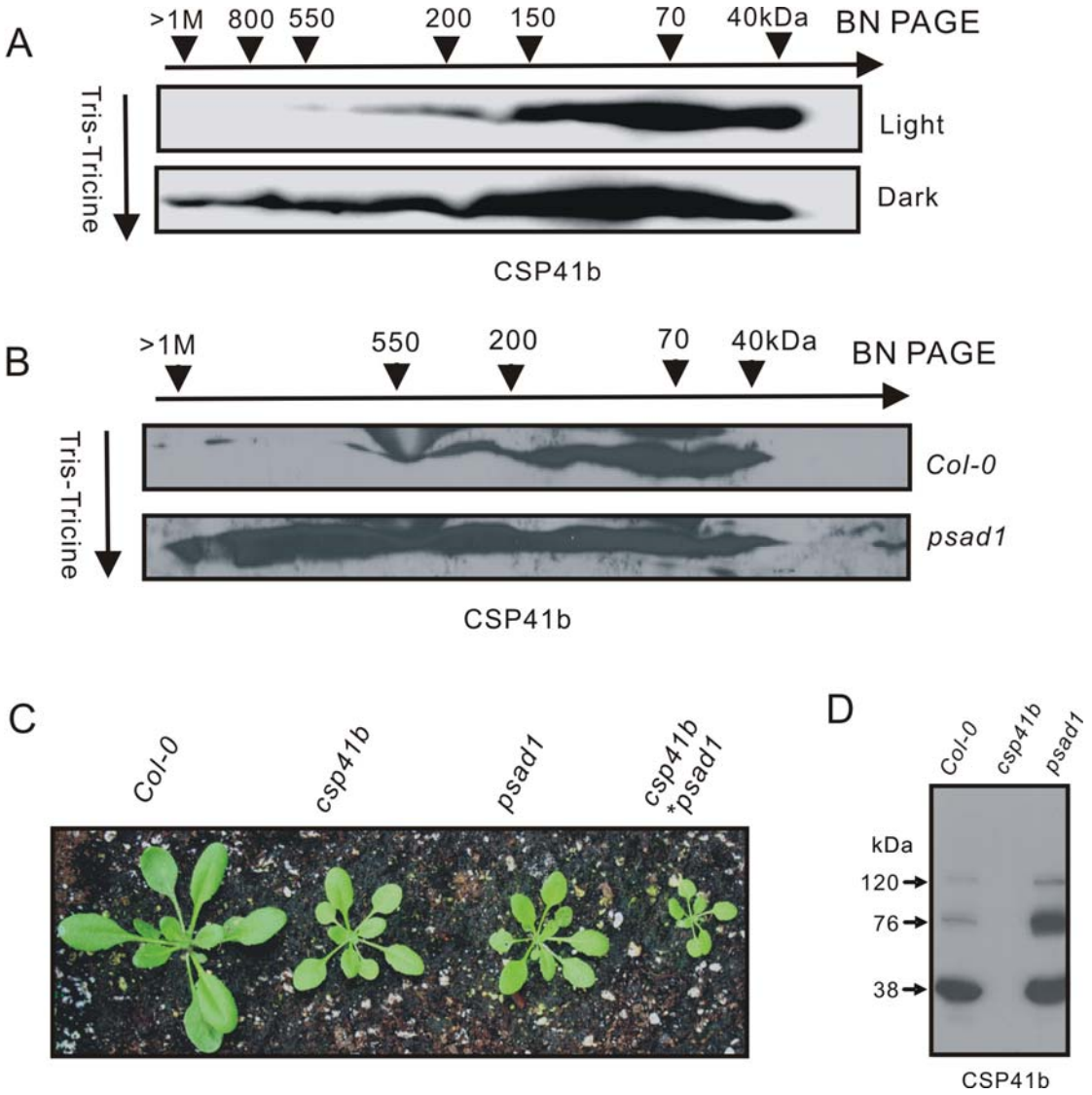


Figure 3.9 CSP41-RNA complexes are regulated by the stomal redox state

(A) CSP41-RNA complexes accumulated in the dark and in the light. For the light treatment, 3-week-old plants were shifted from the dark into ambient light condition 2 hours before chloroplast were isolated. Dark samples were isolated from plants strictly hold under dark conditions

(B) CSP41-RNA complexes accumulated in *psad1* mutants and wild types.

(C) *csp41b* psad1*double mutants show a more severe growth retardation phenotype compared to *csp41b* and *psad1* single mutant lines.

(D) The dimerization of CSP41b in Col-0, *csp41b,* and *psad1*. Stroma (50 µg) from these lines were fractionated on non-reducing SDS PAGE blotted and treated with CSP41b antibodies.

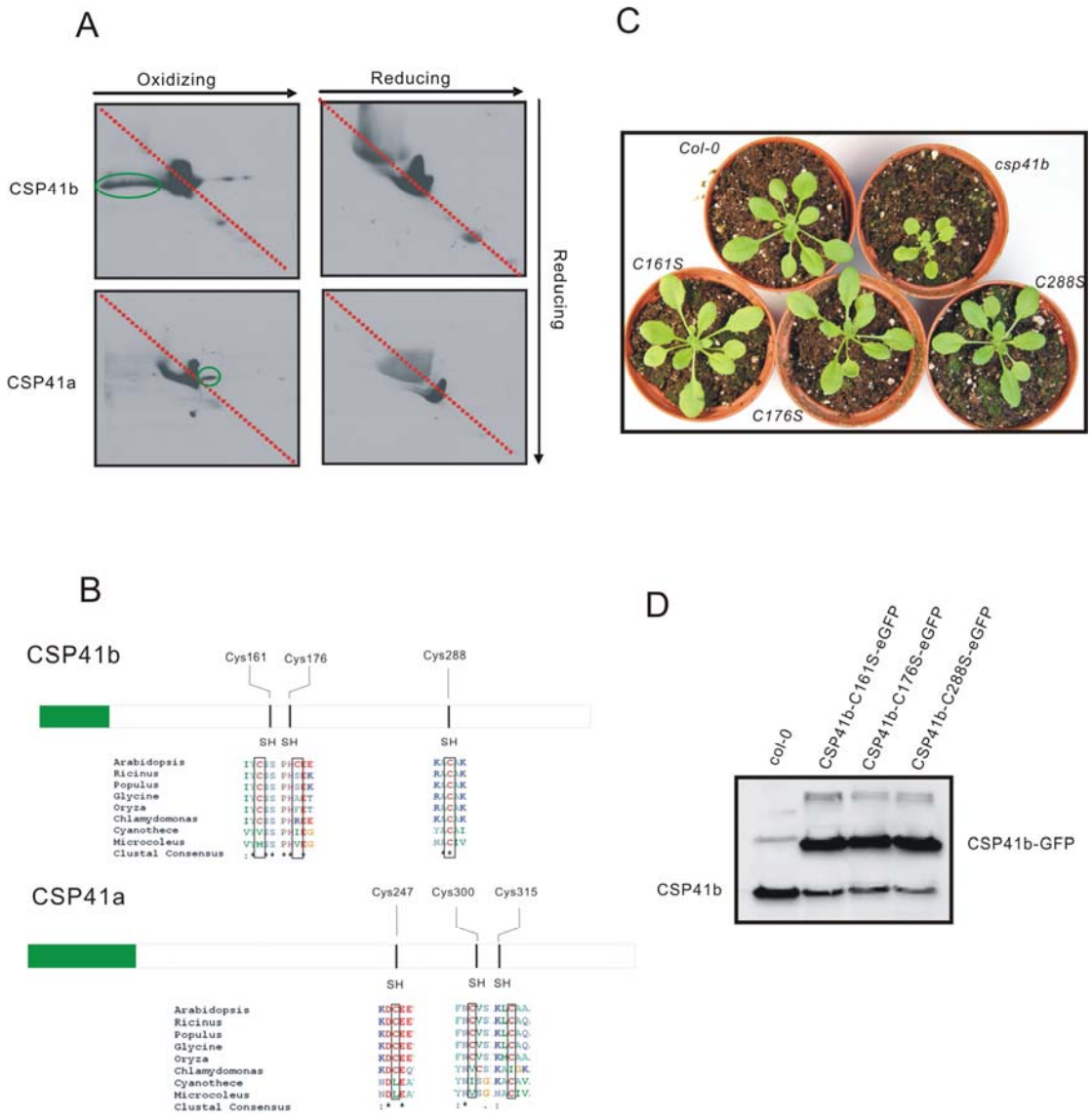


Figure 3.10 Cysteine-serine mutants of CSP41b can complement the *csp41b* phenotype

(A) Diagonal SDS-PAGE to show disulphide bonds in CSP41a and CSP41b. Stroma from Col-0 was treated with diamide (oxidizing) or DTT (reducing) on the first dimension of SDS-PAGE gel. Gel strips cut from both oxidizing and reducing condition were reduced with DTT prior to the second separation. The green circles below and above the diagonal show the intermolecular disulphide bond and intramolecular disulphide bond, respectively.

(B) Cysteines exist in CSP41a and CSP41b. Cysteines in CSP41b were marked as Cys161, Cys176, Cys288; Cysteines in CSP41a were marked as Cys247, Cys300 and Cys315. These cysteines were mutated to serine via overlapping PCR.

(C) Mutagenized C161S, C176S, and C288S variants of CSP41b can complement the *csp41b* phenotype.

(D) Protein levels of CSP41b in Col-0, *CSP41b-C161S-eGFP*, *CSP41b-C176S-eGFP*, and *CSP41b-C288S-eGFP*.

To understand how CSP41-RNA complexes are regulated via disulphide bond formation, cysteines of CSP41a and CSP41b were mutated to serine (Figure 3.10 B). These Cys-Ser mutants were transformed into the *csp41a* and *csp41b* background, respectively. Both CSP41a and CSP41b contain three cysteines. The Cys315 of CSP41a and the Cys288 of CSP41b are conserved in almost all CSP41 homologs. The Cys176 of CSP41b only exists in Arabidopsis. The Cys161 of CSP41b, the Cys247 and the Cys300 of CSP41a are specific for eukaryotic photosynthetic organisms.

Although Cys288 of CSP41b is the most conserved cysteine in all CSP41 homologues from eukaryotic to prokaryotic species, CSP41b-C288S-eGFP, as well as CSP41b-C161S-eGFP and CSP41b-C176S-eGFP, can fully complement the *csp41b* mutant phenotype (Figure 3.10 C). CSP41b-eGFP was equally expressed in these complemented lines as CSP41b in Col-0 (Figure 3.10 D). Since there is no distinct phenotype in the *csp41a* mutant, the phenotype of CSP41a-C247S, CSP41a-C300S and CSP41a-C315S lines in the *csp41a* background are the same like Col-0 (Picture not shown).

3.14 CSP41a and CSP41b can be post-translational modified (PTM).

CSP41a was detected in the chloroplast phosphoproteome in *Arabidopsis* (Reiland *et al.*, 2009). To gain more information about how CSP41-RNA complexes are regulated by post-translational modifications, IEF (Iso-electric focusing) and Western blot analysis were performed to examine modifications of CSP41a and CSP41b. Total stroma from Col-0 and *csp41b* mutants separated on 2D IEF, were blotted to PVDF membranes, followed by Coomassie staining and Western blot analyses with CSP41 antibodies. Western blot signals overlapped with protein spots on the Coomassie stained PVDF membrane (Figure 3.11). Since they can be recognized on Coomassie stained filters, CSP41a and CSP41b are abundant proteins in the stroma. In addition to the main isoform visible by Coomassie staining, other isoforms of CSP41 detected by specific antibodies indicate that CSP41a and CSP41b are post-translational modified. The theoretical p*I* (Iso-electric point) calculated with online software (Bjellqvist *et al.*, 1993; Wilkins *et al.*, 1999) was 6.5 for CSP41a, and 6.8 for CSP41b. The p*I* of CSP41a is lower than that of CSP41b from the 2D IEF gel and the theoretical calculation. By calculation of the Western signal strength, the amount of

the main isoform is around 70% compared to the total amount of CSP41a or CSP41b. The signal strength of the main isoform (Coomassie staining visible spot) of CSP41b and CSP41a is around 3 to 1 on the Coomassie stained PVDF membrane. To get the molecular ratio of CSP41b to CSP41a, this 3:1 ratio was recalculated according to the percentage (70%) of the main isoform and the molecular mass of CSP41 (CSP41a is 36 kDa and CSP41b is 38 kDa). Finally, the molecular ratio of CSP41b to CSP41a range from 2.2 to 2.8 (from 2 independent repetitions).

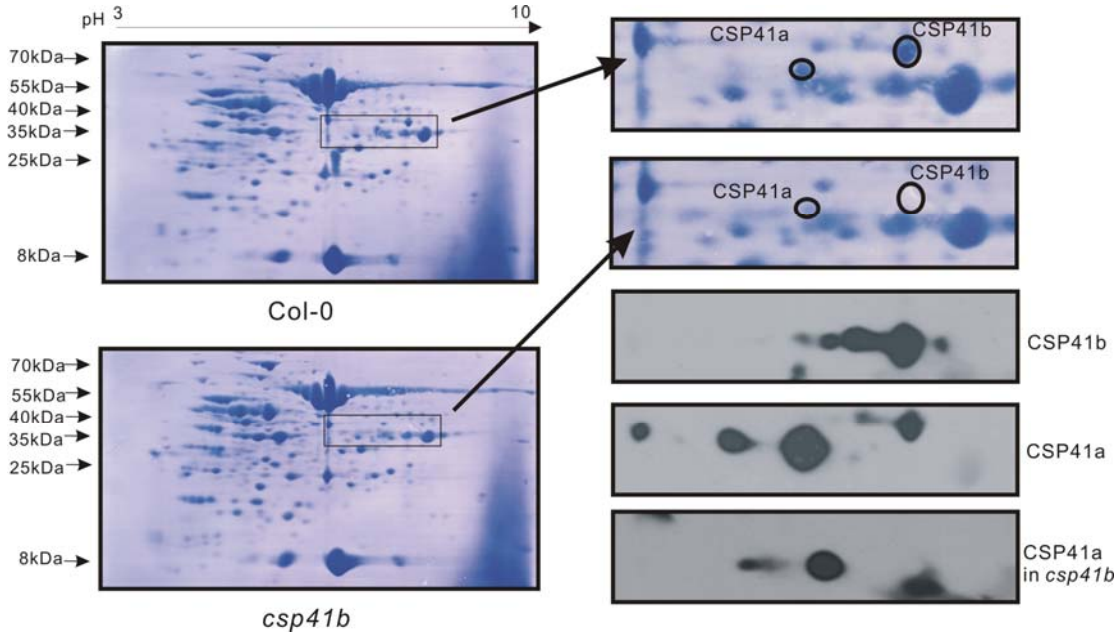


Figure 3.11 CSP41a and CSP41b can be post-translational modified

500 µg stromal proteins from Col-0 and *csp41b* were loaded on the pH 3-10 IEF gel strips. The gel strip was equilibrated with buffers containing DTT followed by buffers containing iodoacetamide, and separated on Tricine-SDS-PAGE for the second dimension. Immunodetected signals of CSP41a and CSP41b overlapped with spots on Coomassie Blue R250 stained membrane. The position of CSP41a and CSP41b are marked by black circles.

3.15 Post-translational modifications of CSP41b differ between dark- and light-adapted plants

The stroma isolated from dark or light adapted plants (as did in Figure 3.9 A) were used for IEF analyses. Stromal proteins from dark and light conditions were differently distributed on the IEF-2D gel shown by Coomassie staining (Figure 3.12

A). The threonine phosphoproteome of the stroma from dark and light detected by the phospho-Thr antibody also shows different patterns to some extent (Figure 3.12 B).

Modifications and abundance of CSP41a were not changed between dark and light condtions. However, the CSP41b level in the dark increases compared to that in the light, which is consistent to the increase of *CSP41b* transcripts to the maximum before nightfall (Hassidim *et al.*, 2007). The level of one CSP41b isoform (spot2) on the acidic end of the main CSP41b isoform (spot3) increases strikingly in the dark compared to that in the light (Figure 3.12 B & D). The observation that spot2 slightly shifted to the acidic end can be explainedby either phosphorylation or acetylation (Zhu *et al.*, 2005). Western blot analyses with the phospho-threonine specific antibody suggest that these CSP41b isoforms (spot1, spot2 and spot3) could be caused by phosphorylation (Figure 3.12 C).

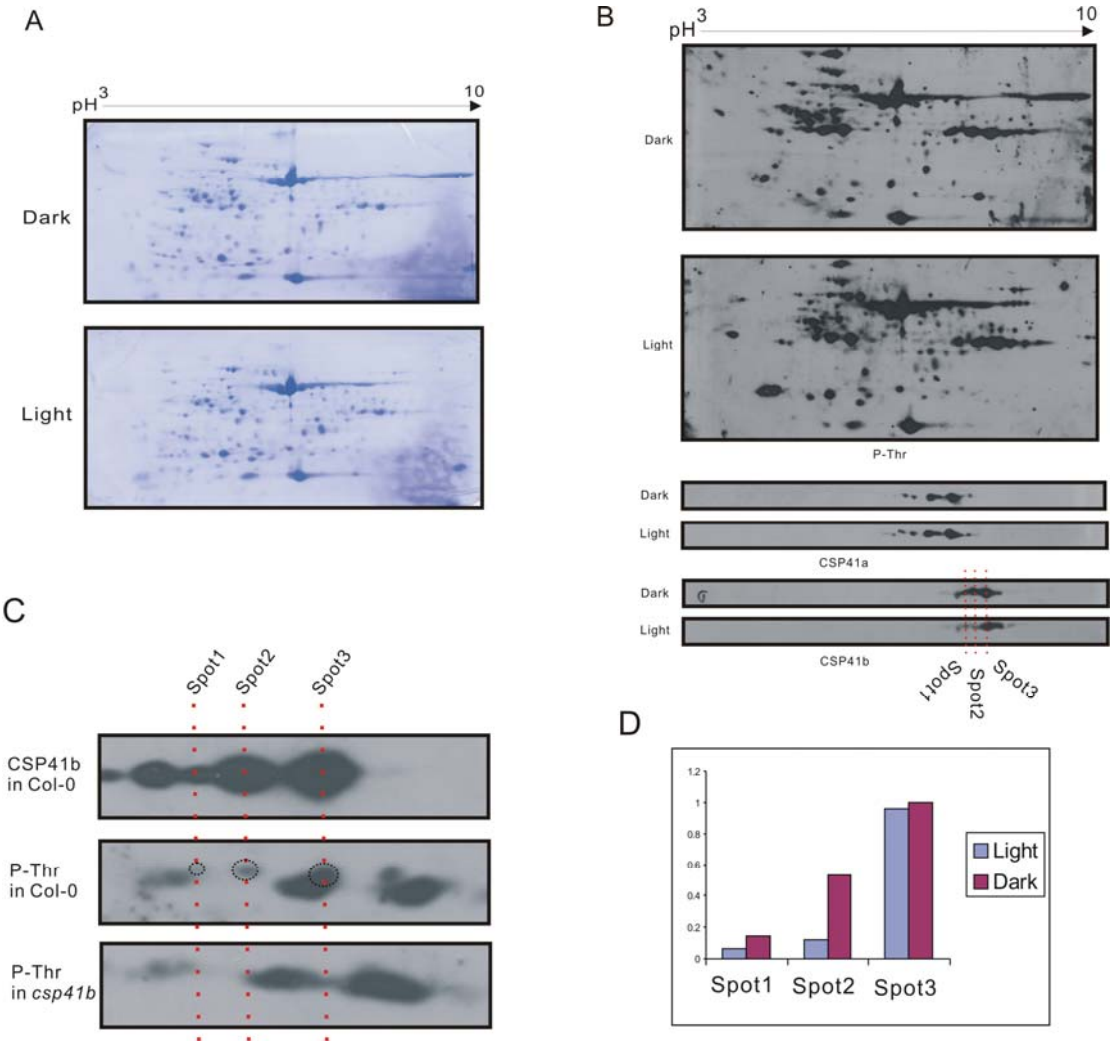


Figure 3.12 Stromal proteins are regulated by post-translational modifications in response to a dark or a light treatment

(A) The stroma isolated from dark and light treated plants were subjected to 2D IEF. The 2nd dimension of gels were blotted to PVDF membranes and stained with Coomassie Blue R250.

(B) The Phospho-Threonine antibody, CSP41a and CSP41b antibodies were used to check the PTM of the stroma isolated from dark and light. The PTM of CSP41a from dark and light are almost the same. However, PTM of CSP41b shows that spot 2 is more pronounced in the dark than that in the light.

(C) Three spots detected by the phospho-Threonine antibody in Col-0 are missing in *csp41b* mutants. Differente CSP41b isoforms are present due to phosphorylated threonine residues. The identity of the spots was proven by the overlay of the immunodetection assay and the phosphopeptide staining assay,.

(D) The signal strength of CSP41b isoforms in figure 3.12 B was calculated. It shows that the intensity of spot2 from the dark is almost increased by a factor of 5.

4 Discussion

4.1 PPP3 can form a small complex in the thylakoid lamellae

PPP3 contains two tentative protein-protein interaction domains (PPI) and a chloroplast transit peptide within its primary sequence. The predicted cTP indicates that PPP3 is a chloroplast protein. PPP3 was confirmed to be a component of the thylakoid membrane through chloroplast fractionation and subsequent Western blot analyses. The results demonstrate that the localization of PPP3 is totally different from that of the PSII complex (PsbD subunit) which is found in the grana fraction. PPP3 is co-localizedwith the PSI complex (PsaD and PsaC subunits) and the $Cytb_6f$ complex (PetC subunit) which are part of the thylakoid lamellae. Although PPP3 is not a component of the photosynthetic complexes, it can form a small complex on the thylakoid membrane. The salt treatments results suggest that PPP3 is attached to the thylakoid lamellae by both hydrophobic and electrostatic interactions. Since both PDZ and TPR domains are able to perform hydrophobic and electrostatic interactions (D'Andrea and Regan, 2003; Liu *et al.*, 2008), it is likely that PPP3 associates to thylakoid membranes through its PPI domains. These data together indicate that PPP3 represents a novel small protein complex attached to the stroma lamellae.

Both PDZ and TPR domain are conserved in all prokaryotes and eukaryotes. However, no other protein containing both these two PPI domains was found in any photosynthetic organisms when performing a BLAST search. Additionally, PPP3 has homologs only in eukaryotes, therefore it is possible either that it is a novel protein evolved and maintained because of a new function in the chloroplast after endosymbiosis, or that it was lost by prokaryotes since they lack chloroplasts.

4.2 What could be the possible biological function of PPP3?

Since none of those *PPP3* knock-out lines shows any distinct phenotype with respect to the wild type neither under normal growth conditions (Greenhouse and climate chamber) nor under stress conditions (Salt stress and drought stress), it is suggested that there could be another chloroplast protein with a redundant function. In the TAIR

database (Swarbreck *et al.*, 2008), the *Arabidopsis* gene locus At5g17170, which is also known as GC4 (Greencut 4) in our lab, is annotated to be related to PPP3. GC4 is a thylakoid membrane protein, which contains a PDZ domain at the N-terminal and a rubredoxin domain at the C-terminal (Peltier *et al.*, 2004). Rubredoxin is a small protein that can bind iron, and participate in electron transfer, sometimes replacing ferredoxin as an electron carrier (Andrews, 2010; Lee *et al.*, 1995). The sequence alignment of these two proteins shows a high similarity between their PDZ domains (Figure 4.1). The generation of *ppp3*gc4* double mutant is still in progress. However, there is no peptide of GC4 detected by MS from the thylakoid Co-IP neither with the PPP3 antibody nor the GFP antibody (Appendix, Table 6.2).

Since PPP3 is phosphorylated in response to wounding (Ishikawa *et al.*, 2005), it is assumed that PPP3 might be involved in chloroplast signaling transduction, which could be complemented by other signaling pathways in *ppp3* mutants.

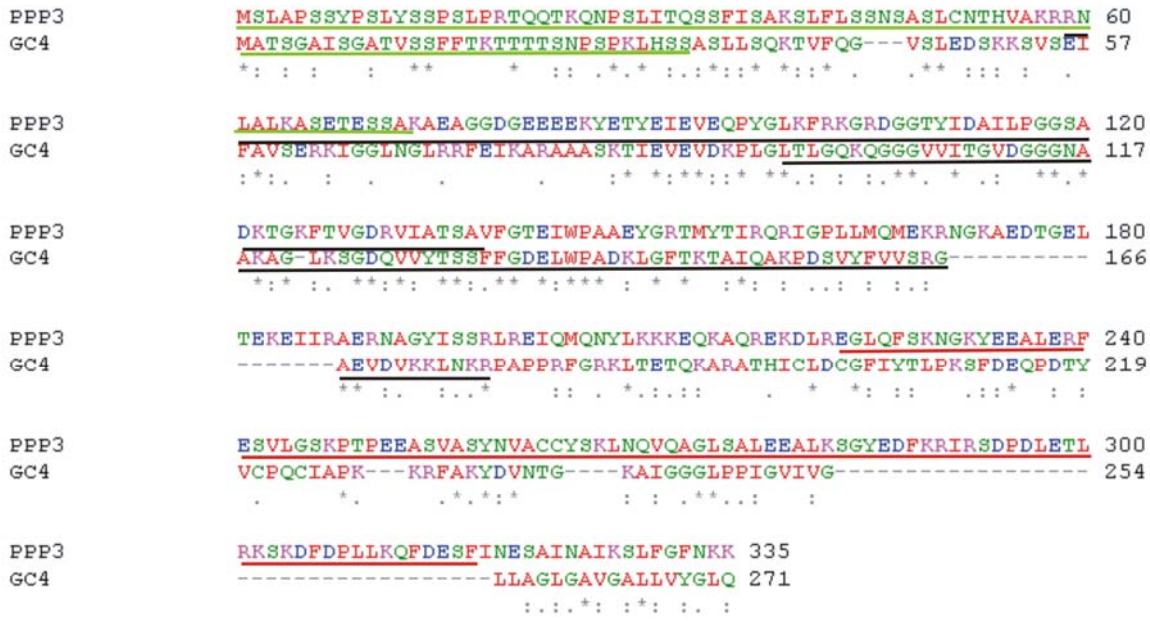


Figure 4.1 Sequences alignment of PPP3 and GC4

Protein alignment shows the similarity of PPP3 and GC4. The underlined sequences show the different domains predicted by ChloroP and InterProScan. The cTPs are represented by green lines, the PDZ domains by black lines, and the TPR domains by red lines.

4.3 What is the biological function of CSP41a and CSP41b?

Since heparin is a negatively charged linear polymer mimicking the single-stranded RNA structure (Capila and Linhardt, 2002), it is commonly used to pull down RNA-binding proteins, such as CSP41a from spinach (Chen *et al.*, 1995). Later, CSP41a was described as an endoribonuclease, belonging to the short-chain dehydrogenase/redutase family (SDR), because CSP41a expressed heterologously in *E.coli* can cleave single- and double-stranded RNA via a domain sharing similarities with the P1 nuclease (Yang *et al.*, 1996; Yang and Stern, 1997). SDR is a large family of enzymes, most of which are known to be NAD(H)- or NADP(H)-dependent oxidoreductases in plants (Persson *et al.*, 2003). The oxidoreductase activity of heterologously expressed CSP41a and CSP41b, however, was not detectable in this study using NADH or NADPH and nitroblue tetrazolium (NBT) as substrates. These data are consistent with the lack in CSP41 of a residue (Tyrosine or Lysine) that is necessary for the oxidoreductase activity (Baker *et al.*, 1998). However, further analyses of CSP41 in the presence of NAD^+ or $NADP^+$ and yet unknown substrates are necessary to completely rule out the possibility of having an oxidoreductase activity.

Reverse genetic approaches and proteome analysis provide opportunities to interpret CSP41 biological functions *in vivo*, but most of the published results are ambiguous and contradictory. For instance, it was reported that CSP41 is a component of the PEP, the 70S ribosome, or pre-ribosome particles in chloroplasts (Pfannschmidt *et al.*, 2000; Yamaguchi *et al.*, 2003; Beligni and Mayfiled, 2008); the CSP41b knock-out *Arabidopsis* lines show same phenotypes like the wild type. The *csp41a*csp41b* double mutant is lethal (as described by Beligni and Mayfiled, 2008), but *csp41b* was reported by other groups to have distinct phenotypes (Hassidim *et al.*, 2007; Bollenbach *et al.*, 2009). The different phenotype of *csp41b* could be explained by the usage of different light conditions (Figure 3.4 A). It is surprising that the phenotype of *csp41a*csp41b* in our group resemble that of *csp41b* instead of being lethal. By Western blot and Northern analyses, it is confirmed that *csp41a* and *csp41b* are complete knock-out lines. We observed that chloroplast transcripts (mRNAs and rRNAs) were decreased with respect to the WT in *csp41b* and *csp41a*csp41b* mutants

in high light conditions (Green house), while they were unmodified under lower light (Climate chamber). The translational defect was detected only in the old leaves of *csp41b* and *csp41a*csp41b,* but not in the young leaves (Figure 3.5). These results indicate that CSP41a and CSP41b are not crucial for the chloroplast translational apparatus, if they are actually components of the 70S ribosome. The severe phenotype of *csp41b* and *csp41a*csp41b* could be due to side effects accumulated during plant growth, since CSP41b is necessary for the plant response to fluctuating light.

To gain insights into the function of the CSP41 proteins, both CSP41a-eGFP and CSP41b-eGFP overexpressor were generated in this study. Surprisingly, CSP41b-eGFP overexpressors show a wild-type phenotype (pictures not shown), but all three independent lines of CSP41a-eGFP overexpressor display a growth retardation phenotype (Figure 3.4 B, C & D). In CSP41a-eGFP overexpressors, the accumulation of CSP41b increases up to 150% and vice versa (Figure 3.4 E & F), which demonstrates that CSP41a and CSP41b can form complexes (Peltier *et al.*, 2006; Bollenbach *et al.*, 2009). The MS data from Co-IP fractions also confirms the association of CSP41a and CSP41b (Appendix, Table 6.1). On the basis of these evidences, it can be assumed that CSP41a and CSP41b have a related function in the chloroplast, as they are associated in a complex. However, the different phenotypes of *csp41a*, *csp41b*, CSP41a-eGFP and CSP41b-eGFP overexpressors, suggest that CSP41a and CSP41b might have only partially redundant functions in *Arabidopsis*.

4.4 CSP41a and CSP41b are RNA-binding proteins required for chloroplast transcripts stabilization *in vivo*

Since the RNA-binding activity was demonstrated *in vitro* using refolded heterologously CSP41a, there is not any solid evidence showing that CSP41a - as well as its homolog CSP41b - can bind RNA *in vivo*. We suggest that both CSP41a and CSP41b are RNA-binding proteins according to the BN-PAGE results (Figure 3.7). By calculating the signal abundance of the high molecular complexes (above 550 kDa), which are confirmed to be CSP41-RNA complexes, it is deduced that around 7% or 6% of the CSP41a and CSP41b proteins could bind RNA. However, it is estimated that around 30% of CSP41a and CSP41b can actually bind RNA, according to Western blot analyses with fractions of a gel filtration performed under native

conditions. The lower RNA-binding ability of CSP41 inferred from the BN PAGE shows that the negative charge of Coomassie can destroy the RNA-CSP41 interaction, based on electrovalent bond. This is also consistent with the fact that the negatively charged heparin can dissociate RNA-CSP41 complexes.

Although Coomassie can destroy RNA-CSP41 complexes, on the BN gel some signals are still visible around 800 kDa or 1 MDa and smears between these spot, which means that CSP41a and CSP41b could have different preferred target RNAs in the chloroplasts. If the molecular size of RNA is estimated according to the average molecular mass of ribonucleotides (about 320 Daltons), the molecular size of CSP41-RNA complexes could range between 76 kDa (binding to a 40 kDa tRNA) and 2.3 MDa (binding to 2.3 MDa rRNA precursor). Based on these calculations, we speculate that the signals of CSP41 from the Western blot of BN-PAGE could comprise multitype complexes. For instance, the most abundant CSP41 signal at 75 kDa could be not only due to dimer of CSP41 (heterodimer of CSP41a and CSP41b or homodimer of CSP41b), but also to small CSP41-RNA complexes. Detection of cpRNPs and ribosomal proteins in the Co-IP fraction of CSP41b-eGFP (Appendix, Table 6.1), indicate that CSP41b might directly interact with these proteins, or that they can be co-precipitated with RNAs that also interact with CSP41b.

Which is the RNA-binding domain of CSP41? Two RNA-binding protein families in chloroplasts employ conserved RNA-binding motifs to bind RNA, such as the PPR and the RRM domains. According to the InterProScan prediction, CSP41 only has the NAD(P)-binding domain (Rossmann fold), since it is a member of the SDR family. NAD or NADP can be bound by the Rossmann fold and used as cofactors. It was suggested that the Rossmann fold is a novel RNA-binding domain of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Nagy *et al.*, 2000). It is speculated that CSP41 could bind RNA via its NAD(P)-binding domain, however, BN-PAGE of stroma treated with NADPH showed no effect on the accumulation of CSP41-RNA complexes (data not shown).

Since CSP41a and CSP41b interact with each other, one aim of this work was to find out if CSP41a or CSP41b bind RNA as monomers or dimers. Early *in vitro* studies showed that CSP41a can bind RNA as a monomer (Yang *et al.*, 1996), and this was

supported by the results of two *in vivo* experiments. First of all, the accumulation of CSP41a was reduced to 20% in the *csp41b* mutant compared to the wild type level, which leads to that CSP41a-RNA complex is undetectable on BN-PAGE. However, overexpressed CSP41a-eGFP in *csp41a*csp41b* double mutants background still can bind RNA. As a second part, overexpression of CSP41a-eGFP in the *csp41a* mutant background, leads to the formation of more CSP41a-RNA complexes than CSP41b-RNA complexes (Figure 3.7 E). These two evidencesindicate that dimerization of CSP41 is not necessary for RNA binding, but it could provide a regulatory pathway to CSP41-RNA complex formation by blocking their putative RNA-binding site through protein-protein interaction.

Since CSP41a has an endoribonuclease activity in *vitro*, it is suggested that CSP41a or CSP41b might be involved in the initiation step of RNA degradation by removing stem-loop structures (Stern *et al.*, 2010). However, more than 30% of CSP41 is involved in RNA-binding in wild type chloroplast. The fact that the transcript abundance is not changed neither in CSP41a-eGFP overexpressors nor in *csp41a* mutants, make it unlikely that CSP41a is involved in RNA degradation, as ribonuclease, *in vivo*. RNA stability assays, further more suggest that CSP41b is required for chloroplast transcripts stabilization in the stroma (Figure 3.8 D).

4.5 What are the RNA targets of CSP41s?

Eight transcripts including six mRNAs (*psaA, psbA, rps18, atpB, rbcL* and *psbC*) and two rRNAs (*rrn16 and rrn23*) from chloroplasts were examined by Slot-blot with RIP fractions (Figure 3.8 A). Both CSP41a and CSP41b show a preference in the target-RNA. This could be interpreted as following: Firstly, CSP41a and CSP41b show different binding affinities to the same transcript, for instance, CSP41b can pull down more *rrn16, rps18,* and *psbA* transcripts, while CSP41a shows a preference for *psbC*. Secondly, CSP41 proteins show a stronger affinity for mRNAs than for ribosomal RNAs, since the enrichment ratios of *rrn23* with CSP41a and CSP41b are lower than 1. Nonetheless, CSP41a and CSP41b can pull down almost the same amount of mRNAs, such as *psaA, atpB*, and *rbcL*. It is still not clear if CSP41 can bind tRNA, which also has secondary stem-loop structures *in vivo*.

The fact that rRNAs are pulled down in RIP fractions can have three explanations: (i), CSP41 binds rRNA precursors directly, but with low affinity. (ii) CSP41 interacts with pre-ribosomal particles (Beligni and Mayfield, 2008), or is a component of the 70S ribosome (Yamaguchi *et al.*, 2003). (iii), the ribosomes containing rRNAs, are bound to mRNA while CSP41b is bound to the stem-loop structure of 3' UTR region of this mRNA. The latter two scenarios are able to explain the fact that rRNAs and ribosomal protein were detectable in the protein fraction of RIP. No matter what the right explanation is *in vivo*, the fact that always more mRNAs than rRNAs can be pulled down, indicates that the binding of CSP41a and CSP41b to mRNA might be the initial step necessary for subsequent activities.

How can CSP41a and CSP41b recognize different transcripts? Early experiments have shown that an AU-rich *cis*-element next to the 3' UTR stem-loop is necessary for binding RNA *in vitro* (Chen *et al.*, 1995). CSP41a prefers to bind the 3' UTR region of *petD* rather than those of *psbA* and *rbcL*, because the latter miss this AU-rich *cis*-element (Yang and Stern, 1997). However, CSP41a and CSP41b can bind these transcripts *in vivo*. This could be explained by the structural differences of the mRNAs (only the 3' UTR region used for the *in vitro* experiments, but full-length mRNAs) and different CSP41 proteins (refolded recombinant protein expressed in bacteria in the first case, but natural protein in the second). This indicates that either the AU-rich element is not required for RNA binding *in vivo*, or CSP41a and CSP41b can generally bind stem-loop structures, and their affinity for transcripts depends on the length of the stem-loop. The hypothesis that CSP41b can generally bind the stem-loop structure of RNA is supported by two facts: (i), CSP41a can be pulled down by heparin (Chen *et al.*, 1995) and heparin can dissociate the CSP41-RNA complexes (Figure 3.7 A). (ii), heparin without any *cis*-element has strong affinity for CSP41a and CSP41b. Further experiments to identify the sequence regions bound and covered by CSP41a and CSP41b from these RIP fractions are necessary.

4.6 How is CSP41-RNA complex regulated?

The light dependent phenotype of *csp41b* implies that CSP41b might play regulatory roles in response to fluctuating light conditions. Changes of light lead to changes of the redox state in the stroma. The following evidences suggest that CSP41-RNA

complexes are regulated by the redox state: (i), it was shown that the transcription of CSP41b is under circadian control with a peak of mRNA level in the evening (Hassidim *et al.*, 2007). This is consistent with the results of Western analyses, indicating that the CSP41b protein levels were increased around 20% in the dark with respect to that in the light. (Figure 3.9 A, Figure 3.12 B). (ii), dark adapted wild type plants contain more CSP41b-RNA complexes than light adapted wild type plants (Figure 3.9 A), and the same phenomenon is also observed in the *psad1* mutant, which contains more CSP41b-RNA complexes than Col-0. (iii), the double mutant of *csp41b*psad1* shows a more pronounced growth retardation phenotype (Figure 3.9 C), and the CSP41b dimers in *psad1* accumulated more than that in Col-0 (Figure 3.9 D). The CSP41b-RNA complex and the CSP41b protein are accumulated more in the oxidized stroma, which implies that they are regulated, directly or indirectly, by the redox state.

The direct regulation of the CSP41-RNA complex is probably achieved by regulating the thiol groups of the CSP41 proteins. CSP41a and CSP41b form intra-molecular and inter-molecular disulphide bond, respectively, and those provide the possibility of direct redox regulation (Ströher and Dietz, 2008, Figure 3.10 A). However, the fact that all the Cys-Ser CSP41b-eGFP mutants can complement the *csp41b* phenotype, indicates that none of these cysteine mutations (including that of the most conserved, C288S) can abrogate the biological function of CSP41b, and that the CSP41b-RNA interaction does not rely on the dimerization of CSP41b caused by disulphide bridge. These data indirectly support the idea that CSP41 dimers are not essential for RNA-binding, as discussed in Part 4.4. To interpret how CSP41a-RNA complexes are regulated by the intra-molecular disulphide bond, studies on Cys-Ser mutated CSP41a is still necessary.

The redox regulation of the CSP41-RNA complexes can be mediated indirectly by other post-translational modifications, such as phosphorylation. Both CSP41a and CSP41b are phosphorylated (Figure 3.12 C, Reiland *et al.*, 2009), and CSP41b shows different PTM patterns in dark and light conditions (Figure 3.12 B). These results suggest the possibility of phosphorylation regulation of the CSP41-RNA complex. However, the PTM patterns for CSP41a in the dark and in the light are almost the same. Together with the complemented phenotype of all the Cys-Ser CSP41b-eGFP,

these results suggest that the CSP41b-RNA complex could be regulated indirectly by the stromal redox state via phosphorylation, and the CSP41a-RNA complex directly by the stromal redox state via thiol groups. To confirm these hypotheses, it is necessary to detect which threonine residues are phosphorylated.

4.7 The divergence of CSP41a and CSP41b during evolution.

The endoribonulease activity of CSP41a will not be discussed in this section, since it was only shown *in vitro* using a recombinant CSP41a expressed in bacteria and refolded. Many evidences support the idea that CSP41a and CSP41b have different functions, even though they are both RNA-binding proteins: (i), phylogenetic analysis shows that only one homologous gene (Slr1540) is present in cyanobacteria, but two (CSP41a and CSP41b) in the photosynthetic eukaryotes. These suggest that the two isoforms diverged during evolution to achieve different functions (Figure 4.2); (ii), analyses of transcriptome suggest that CSP41b, not CSP41a, falls into the regulon 2 (Biehl *et al.*, 2005); (iii), the *csp41b* mutant shows a different phenotype compared to the *csp41a* mutant, and the CSP41b overexpressor shows a different phenotype with respect to the CSP41a overexpressor; (iv), CSP41a-eGFP overexpressed in the *csp41a*csp41b* double mutant background could not complement the *csp41b* phenotype; (v), CSP41a and CSP41b display a different RNA-binding preference; (vi), one additional band containing CSP41a-eGFP (higher molecular weight complex) can be detected in BN-PAGE (Figure 3.7 C). More surprisingly, this CSP41a-eGFP complex around 1 MDa is also found to be attached to the thylakoid membrane, which indicates that CSP41a might has an additional function of transfer mRNA onto thylakoid membranes for translation.

To investigate if the CSP41 homolog in cyanobacteria can complement the CSP41 function in higher plants, the Slr1540 protein was fused to the transit peptide of CSP41a and CSP41b, respectively, and transformed into the *csp41b* mutant. However, no positive cTP_{CSP41a}-Slr1540-eGFP transformant was obtained, and several positive cTP_{CSP41b}-Slr1540-eGFP transformants were easily silenced at T1 generation. These results imply that CSP41b might not have the same function of its cyanobacterial homolog. To interpret the different functions of eukaryotic CSP41a and CSP41b, it is

necessary to replace the *Synechocystis* Slr1540 with CSP41a or CSP41b for further studies.

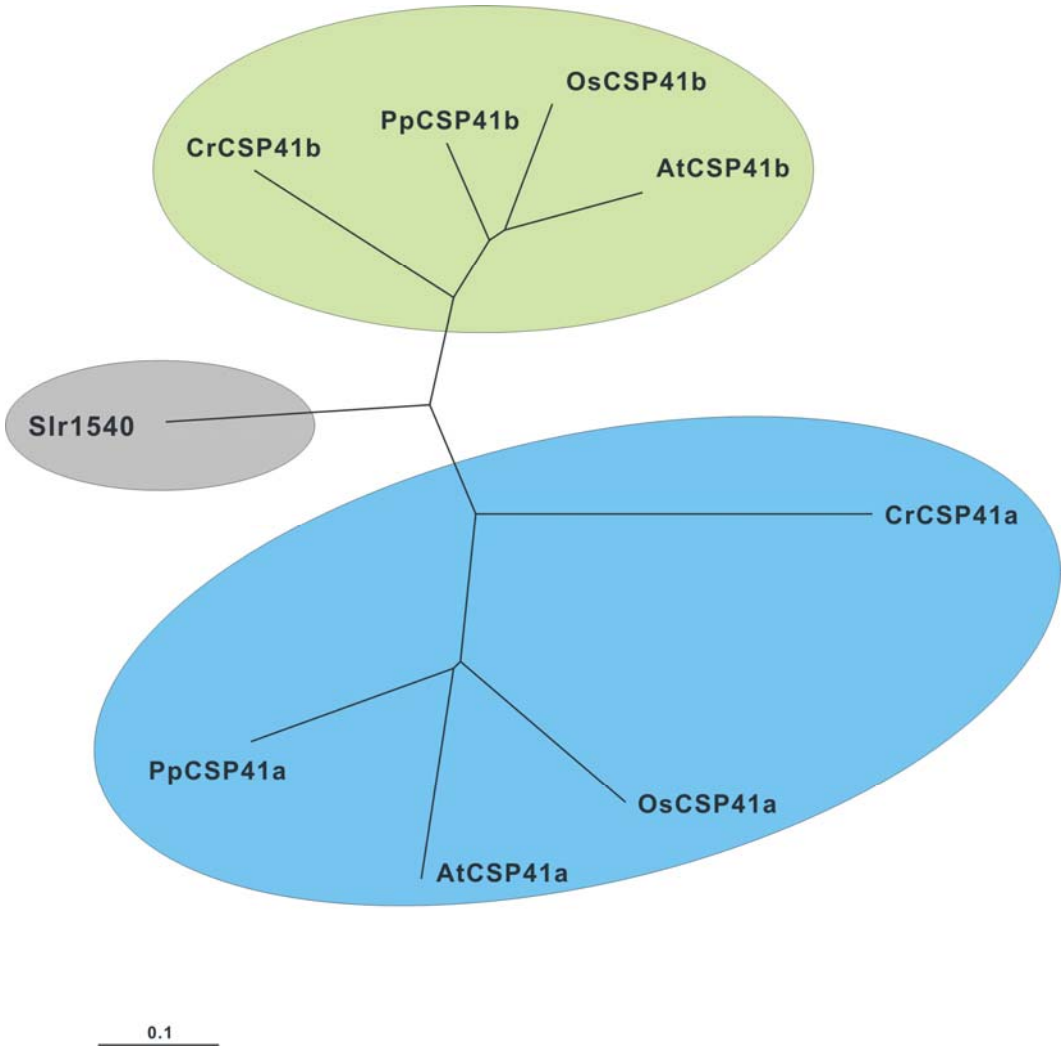


Figure 4.2 Divergence of CSP41a and CSP41b family

The phylogenetic tree of CSP41 protein family is built with CSP41 homologs obtained from different organisms by BLAST search against the protein databases, such as *Chlamydomonas reinhardtii* (Cr), *Oryza Sativa* (Os), *Arabidopsis thaliana* (At), *Physcomitrella patens* (Pp). Slr1540 is the only CSP41 homolog in *Cyanobacteria* (*Synechocystis sp. PCC6803*). The eukaryotic CSP41 homologs can be divided into the CSP41a subfamily (blue) and the CSP41b subfamily (green). Both of these subfamilies diverged from the Cyanobacterial ancestor Slr1540, during evolution.

4.8 The regulatory model of CSP41-RNA complexes between dark and light

To conclude, a model about the regulation of CSP41-RNA complexes (Figure 4.3) is summarized. The proposed regulatory mechanism consists of the following steps: (i), in the light, CSP41a and CSP41b are not accessible to RNA, therefore ribosomes start to translate mRNA; (ii), in the dark, ribosomes are released from mRNA, and CSP41 proteins are regulated by the stromal redox state probably via phosphorylation to protect RNA from RNase degradation; (iii), CSP41a and CSP41b bind RNA as monomers. In this model, the proposed biological function of CSP41a and CSP41b is to stabilize mRNA during the night until their light-dependent translation starts. This protective mechanism can provide an economic way for plants to maintain transcripts a certain level, rather than synthesize them *de novo*.

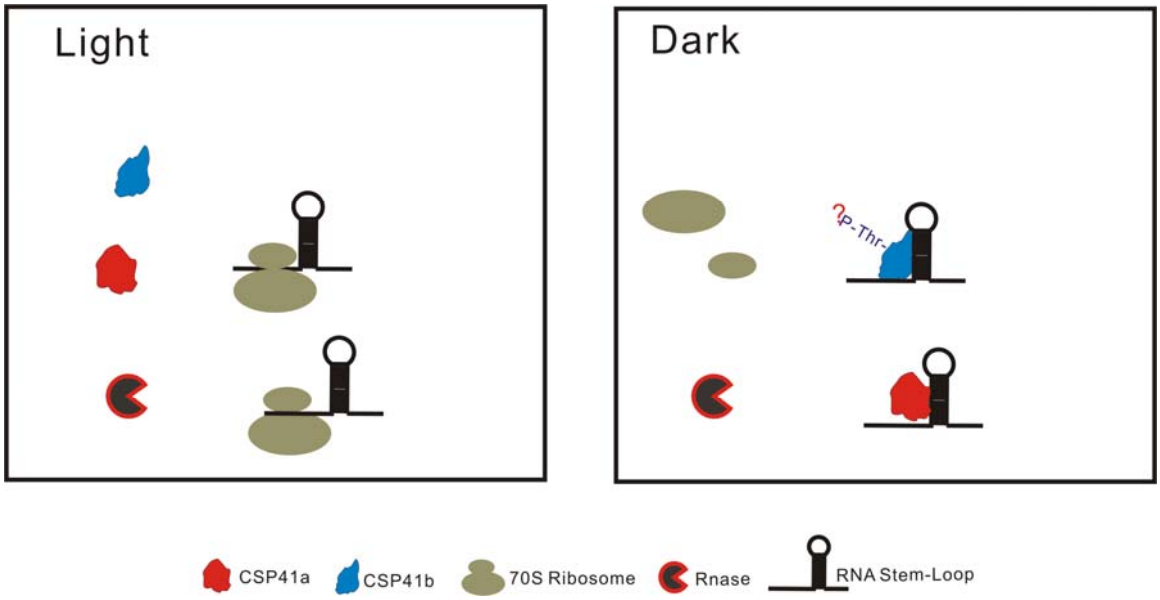


Figure 4.3 Model about how CSP41-RNA complexes are regulated

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6 Appendix (Mass spectrometry sequencing data)

CSP41a-eGFP

Reference	Annotation	Peptide	(Hits)
AT3G63140.1	**CSP41a**	**446**	**(444 2 0 0 0)**
AT1G09340.1	**CSP41b**	**50**	**(50 0 0 0 0)**
AT5G58430.1	putative exocyst subunits	3	(2 1 0 0 0)
AT1G50940.1	the electron transfer flavoprotein ETF alpha	2	(1 1 0 0 0)
ATCG00800.1	chloroplast ribosomal protein S3	2	(2 0 0 0 0)

CSP41b-eGFP

Reference	Annotation	Peptide	(Hits)
AT1G09340.1	**CSP41b**	**274**	**(274 0 0 0 0)**
AT3G63140.1	**CSP41a**	**29**	**(29 0 0 0 0)**
AT3G63490.1	Ribosomal protein L1p/L10e family	11	(11 0 0 0 0)
AT1G55490.1	beta subunit of the chloroplast chaperonin 60	6	(6 0 0 0 0)
AT1G07320.1	ribosomal protein L4	6	(6 0 0 0 0)
AT4G09040.1	RNA-binding (RRM/RBD/RNP motifs) family protein	5	(5 0 0 0 0)
AT2G28000.1	alpha subunit of the chloroplast chaperonin 60	4	(4 0 0 0 0)
AT1G35680.1	Ribosomal protein L21	4	(4 0 0 0 0)
ATCG00800.1	chloroplast ribosomal protein S3	4	(4 0 0 0 0)
ATCG00380.1	ribosomal protein S4	4	(4 0 0 0 0)
AT1G05190.1	structural constituent of ribosome, rRNA binding;	4	(4 0 0 0 0)
AT1G01820.1	member of the peroxin11 (PEX11) gene family	4	(4 0 0 0 0)
ATCG00830.1	ribosomal protein L2	2	(2 0 0 0 0)
AT2G35410.1	RNA-binding (RRM/RBD/RNP motifs) family protein	2	(2 0 0 0 0)
AT3G27850.1	50S ribosomal protein L12-C	1	(1 0 0 0 0)
AT2G33800.1	Ribosomal protein S5	1	(1 0 0 0 0)
ATCG00770.1	RIBOSOMAL PROTEIN S8	1	(1 0 0 0 0)
AT5G26742.1	DNA/RNA helicase	1	(1 0 0 0 0)
AT5G30510.1	ribosomal protein S1	1	(1 0 0 0 0)

Table 6.1 Putative pull-down proteins by CSP41a-eGFP and CSP41b-eGFP detected by mass spectrometry sequencing

Proteins are pulled-down by CSP41a-eGFP and CSP41b-eGFP. AGI number marked red shows CSP41a and CSP41b are most abundant co-purified proteins in CSP41b-eGFP and CSP41a-eGFP, respectively. AGI numbers marked green show ribosomal proteins.

GFP antibody			
Reference		Peptide (Hits)	
Scan(s)	Peptide	Ions	
AT1G55480.1	PPP3	30	(30 0 0 0 0)
ATCG00120.1	ATPA	6	(6 0 0 0 0)
ATCG00280.1	PSBC	4	(4 0 0 0 0)
AT1G55480.1	PPP3	4	(4 0 0 0 0)
ATCG00720.1	PETB	2	(2 0 0 0 0)
ATCG00270.1	PSBD	2	(2 0 0 0 0)
AT4G04640.1	ATPC1	1	(1 0 0 0 0)
AT5G08670.1	hydrogen ion transporting ATP synthase	1	(1 0 0 0 0)

PPP3 antibody			
Reference		Peptide (Hits)	
Scan(s)	Peptide	Ions	
AT1G55480.1	PPP3	10	(6 0 0 0 0)
AT3G15720.1	glycoside hydrolase family 28 protein	1	(1 0 0 0 0)

Table 6.2 Putative pull-down proteins by PPP3-eGFP detected by mass spectrometry sequencing

Acknowledgements

Firstly, I would like to thank Prof. Dr. Dario Leister for giving me the opportunity to perform my Ph.D. research in his research group.

Additionally, I would like to thank Dr. Ute Armbruster and Dr. Thilo Rühle, for their supervision, and willing to help in the past 3 years.

I would like to thank Alexander Hertle for helping and discussing in my projects, Julia Davidova and Mathias Pribil for performing the MS analyses, Rob Sharwood and David Stern for providing antibodies against CSP41a and CSP41b, Lin from AG Nickelsen for providing the synechocystis DNA, Csaba Koncz for allowing me to screen his T-DNA insertion library and Ralf Bernd Kloesgen for providing cTP_{FNR}:eGFP seeds.

I would like to thank Dr Thilo Rühle, Dr. Ute Armbruster, Stefania Viola, Alexander Hertle, Wentent Xu and Wang Fei for reading and correcting my dissertation.

I would like to thank other colleagues in this nice researching team, who are always willing to discuss experimental problems or novel hypotheses.

I would like to thank my friends in the soccer team, who give me the best summer time every Monday and Wednesday.

I would like to thank my parents and Fei, for supporting and helping me in the stressful Ph.D. time.

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Ehrenwörtliche Versicherung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel und Quellen angefertigt habe. Die Autorin hat zuvor nicht versucht, anderweitig eine Dissertation einzureichen oder sich einer Doktorprüfung zu unterziehen. Die Dissertation wurde keiner weiteren Prüfungskommission weder in Teilen noch als Ganzes vorgelegt.

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