Linköping University Medical Dissertations No. 1296

Reversible modifications of chloroplast proteins and assessment of their functions

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Linköping 2012

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Cover design: Jörgen Woss and Björn Ingelsson

ISBN: 978-91-7519-952-8
ISSN: 0345-0082
Printed by Liu-Tryck, Linköping 2012

-Blå!

Björn Ingelsson, 11 år, i den radiosända frågesporttävlingen Skolfiket som svar på utslagsfrågan:

Vilken färg har klorofyll?

ABSTRACT

Oxygenic photosynthesis is the process of solar energy conversion into chemical energy in the form of carbohydrates. This event is carried out by plants, algae and cyanobacteria and represents the starting point of the food chain in which most organisms are fed. Due to never-ending changes in the surrounding environment, these photoautotrophic organisms have evolved different acclimatizing strategies to optimize photosynthesis. Many of these fine-tuning mechanisms are dependent on reversible modifications of proteins on a post-translational level. In my research I have been focused on such reversible modifications of proteins in the organelle where photosynthesis takes place – the chloroplast – using the model plant *Arabidopsis thaliana*.

Within chloroplasts, light-driven reactions of photosynthesis are catalyzed by several multi-subunit protein complexes in the thylakoid membrane. Proteins need to be folded properly in order to function correctly. A rate-limiting step of protein folding is the isomerization of the peptide bond around proline, a step that is catalyzed by enzymes possessing peptidyl-prolyl *cis-trans* isomerase (PPIase) activity. Within the thylakoid lumen, only two proteins have been found to possess PPIase activity, FKBP13 and CYP20-2. Both these enzymes belong to a protein superfamily called immunophilins - ubiquitous proteins attributed with several different functions. By characterization of Arabidopsis mutants lacking FKBP13 and CYP20-2 I found that PPIase activity is a dispensable function of immunophilins in the thylakoid lumen.

A common post-translational modification of chloroplast proteins is phosphorylation. Protein phosphorylation alters protein functions and is a reversible mechanism utilized by plants for rapid acclimation to changes in the incident light. These events require the action of kinases and phosphatases that either add or remove phosphate groups on proteins, respectively. I have characterized mutants deficient in protein phosphatases responsible for dephosphorylation of thylakoid proteins. These phosphatases, PPH1 and PBCP, represent key players in acclimation of the photosynthetic machinery to changes in light quality/quantity. In addition, I discovered that phosphorylation of pTAC16, a protein associated with the chloroplast gene-expression machinery, depends on the presence of STN7; a light-regulated protein kinase located in the thylakoid membrane. This finding could provide a link between the redox state of the photosynthetic apparatus and chloroplast gene expression.

POPULÄRVETENSKAPLIG SAMMANFATTNING

I fotosyntesen omvandlar växter, alger och vissa bakterier solljus till energi i form av kolhydrater via en kedja av biokemiska reaktioner. Som biprodukt formas även det livsnödvändiga syret och därför spelar fotosyntesen en central roll för hur livet på jorden ser ut.

För den fotosyntetiska organismens överlevnad är det helt avgörande hur väl den kan ta tillvara på solljuset. För mycket ljus skadar de proteiner som katalyserar reaktionerna i fotosyntesen samtidigt som för lite ljus kräver att fotosyntesen fungerar optimalt för att göra det bästa möjliga av det ljus som finns att tillgå. Eftersom en växt inte kan flytta sig från en plats till en annan för att få bättre förhållanden måste den istället anpassa fotosyntesen via modifieringar av den fotosyntetiska apparaten.

Min forskning har haft som mål att öka förståelsen för hur dessa finjusteringar går till i modellväxten *Arabidopsis thaliana* (backtrav). Modifiering av fotosyntetiska proteiner genom fosforylering är ett vanligt sätt att ändra ett proteins egenskaper på och samtidigt anpassa dess funktion till aktuella förhållanden. Reversibel proteinfosforylering kräver ett samspel mellan de två enzymgrupperna kinaser och fosfataser som sätter dit eller tar bort fosfatgrupper från proteiner. I mitt avhandlingsarbete har jag identifierat och karakteriserat fosfataset PPH1 som krävs för att växten ska kunna balansera och optimera sitt ljusinfångande. Jag har även varit delaktig i karaktäriseringen av ytterligare ett fosfatas, PBCP. I motsats till PPH1 är PBCP inriktat mot de proteiner som kallas fotosyntesens kärnproteiner vars fosforylering påverkar både reparationen av den fotosyntetiska apparaten och de fotosyntetiska membranens struktur. Växter kan också anpassa fotosyntesen till nya förhållanden genom ändringar i proteiners genuttryck och även här spelar fosforyleringar en viktig roll. Sedan tidigare är det känt att kinaset STN7 är inblandat i sådana förändringar men hur kopplingen mellan STN7 och ändring i genuttryck ser ut är okänt. I mitt arbete har jag upptäckt att fosforylering av proteinet pTAC16 är direkt beroende av STN7. pTAC16 är en del av kloroplastens egna genmaskineri vilket gör pTAC16 en god kandidat till att vara en länk mellan STN7 och genreglering. Vid sidan av proteinfosforyleringar har jag även studerat en grupp av proteiner som kallas immunofiliner. De har historiskt sett förmodats vara inblandade i veckningen av proteiners tredimensionella struktur då många immunofiliner har så kallad PPIas-aktivitet och kan katalysera vridningen av

bindningar innehållande aminosyran prolin – ett annars långsamt steg i proteinveckningen. Genom att analysera växter som saknar denna katalytiska förmåga i fotosyntetiska membran kom jag fram till att PPIas-aktivitet inte utgör den primära funktionen för immunofiliner i dessa membran vilket motsäger det man tidigare trott.

LIST OF PAPERS

This thesis is based on the following papers which are referred to in the text by their roman numerals:

I. **Ingelsson, B., Shapiguzov, A., Kieselbach, T. and Vener, A.V. (2009).** Peptidyl-prolyl isomerase activity in chloroplast thylakoid lumen is a dispensable function of immunophilins in *Arabidopsis thaliana*. Plant Cell Physiol 50, 1801-14.

II. **Shapiguzov, A.*, Ingelsson, B.*, Samol, I., Andres, C., Kessler, F., Rochaix, J.D., Vener, A.V. and Goldschmidt-Clermont, M. (2010).** The PPH1 phosphatase is specifically involved in LHCII dephosphorylation and state transitions in *Arabidopsis*. Proc Natl Acad Sci U S A 107, 4782-7.

*A.S and B.I contributed equally to this work.

III. **Samol, I., Shapiguzov, A., Ingelsson, B., Fucile, G., Crèvecoeur, M., Vener, A.V., Rochaix, J. D., and Goldschmidt-Clermont, M.** The role of PHOTOSYSTEM II CORE PHOSPHATASE in light acclimation of photosynthesis in *Arabidopsis*. Submitted to The Plant Cell 2012.

IV. **Ingelsson, B., and Vener, A.V. (2012).** Phosphoproteomics of *Arabidopsis* chloroplasts reveals involvement of the STN7 kinase in phosphorylation of nucleoid protein pTAC16. FEBS Letters, in press.

Other publications

Strid, T., Svartz, J., Franck, N., Hallin, E., Ingelsson, B., Söderström, M. and Hammarström, S. (2009). Distinct parts of leukotriene C(4) synthase interact with 5-lipoxygenase and 5-lipoxygenase activating protein. Biochem Biophys Res Commun 381, 518-22.

TABLE OF CONTENTS

ABSTRACT VII

POPULÄRVETENSKAPLIG SAMMANFATTNING IX

LIST OF PAPERS XI

ABBREVIATIONS XIV

1. INTRODUCTION 15

1.1. Oxygenic photosynthesis 16
1.1.1. Acclimation mechanisms 17
1.2. Immunophilins – a ubiquitous protein family 19
1.2.1. Chloroplast immunophilins 21
1.3. Reversible protein phosphorylation in chloroplasts 23
1.3.1. STN8-dependent phosphorylation 23
1.3.2. STN7-dependent phosphorylation 24
1.3.3. Regulation of STN7 24
1.3.4. Other chloroplast kinases 25
1.3.5. Protein phosphatases 25
1.3.6. Dephosphorylation of thylakoid proteins 26
1.4. State transitions 28
1.5. Redox control of gene expression 30

2. METHODOLOGIES 31

2.1. *Arabidopsis thaliana* as a model organism 31
2.2. PPIase activity measurements 32
2.3. Mass spectrometry 33
2.3.1. Enrichment and quantification of phosphorylated peptides 35

3. AIMS OF THE RESEARCH 37

4. MAJOR FINDINGS 38

4.1. Characterization of Arabidopsis mutants deficient in lumenal PPIase activity (Paper I) 38
4.1.1. PPIase activity depletion in the *fkbp13* mutant 38
4.1.2. Characterization of the *fkbp13/cyp20-2* mutant depleted in lumenal PPIase activity 39
4.1.3. FKBP13 and Rieske protein 40
4.1.4. PPIase activity is a dispensable function of immunophilins in the thylakoid lumen 41

4.2. Identification of two protein phosphatases involved in dephosphorylation of thylakoid proteins (Paper II & III) 42
4.2.1. Characterization of the *pph1* mutant deficient in LHCII dephosphorylation (Paper II) 42
4.2.2. PPH1 in state transitions 44
4.2.3. Identification of a mutant deficient in dephosphorylation of PSII core proteins (Paper III) 45
4.2.4. Characterization of the *pbcp* mutant 47

4.3. Comparative phosphoproteomic analysis of Arabidopsis wild type and mutant plants deficient in STN7 and STN8 (Paper IV) 48
4.3.1. Sample preparation 48
4.3.2. Identification of phosphopeptides 49
4.3.3. Distribution of pTAC16 51

5. SUMMARY OF MAJOR FINDINGS 53

6. CONCLUDING REMARKS 55

7. ACKNOWLEDGEMENTS 56

8. REFERENCES 58

ABBREVIATIONS

Chla	Chlorophyll a
CID	Collision induced fragmentation
CK2	Casein kinase 2
CsA	Cyclosporin A
CSK	Chloroplast sensor kinase
ESI	Electrospray ionization
ETD	Electron transfer dissociation
FKBP	FK506-binding proteins
IMAC	Immobilized metal-affinity chromatography
LC	Liquid chromatography
LHCII	Light harvesting complex of photosystem II
LTR	Long-term response
MALDI	Matrix-assisted laser desorption ionization
NDH	NAD(P)H dehydrogenase complex
NPQ	Non-photochemical quenching
OEC	Oxygen evolving complex
PC	Plastocyanin
PEP	Plastid-encoded RNA polymerase
PPIase	Peptidyl-prolyl *cis-trans*-isomerase
PQ	Plastoquinone
PQH_2	Plastoquinol
PSI	Photosystem I
PSII	Photosystem II
TAK	Thylakoid-associated kinase

1. INTRODUCTION

The ability to use the Sun as the only source to meet the demand for energy is an ability restricted to plants, algae and some bacteria – photoautotrophic organisms able to convert carbon dioxide into organic compounds to be used as food for the organism. This process, called *photosynthesis*, is a prerequisite for most life on Earth and can be summarized in the following equation where (CH_2O) represents carbohydrates:

$$\mathbf{CO_2 + H_2O + Light \longrightarrow (CH_2O) + O_2}$$

The energy produced is used for the organism's growth but also acts as the starting point for the food chain in which other organisms feed. In addition, as seen in the equation, oxygen, essential for many life forms, is formed as a side product making photosynthetic organisms indispensable for life as we know it.

Photosynthesis takes place within an organelle unique to photosynthetic organisms, the chloroplast (**Figure 1**). The chloroplast is built up by an inner and outer envelope enclosing a soluble milieu called the stroma and a third membrane system called the thylakoid membrane. The thylakoid membrane is the site of photosynthesis where light is harvested, water molecules are spilt and electrons are used for formation of compounds used in the stroma for carbon dioxide assimilation and sugar production. Thylakoids are differentiated into two distinct membrane regions: the stacked (or "appressed") grana and the interconnecting single-membrane region stroma lamellae. The fluid compartment enclosed by the thylakoids is known as the lumen (Anderson and Andersson 1988; Nelson and Ben-Shem 2004).

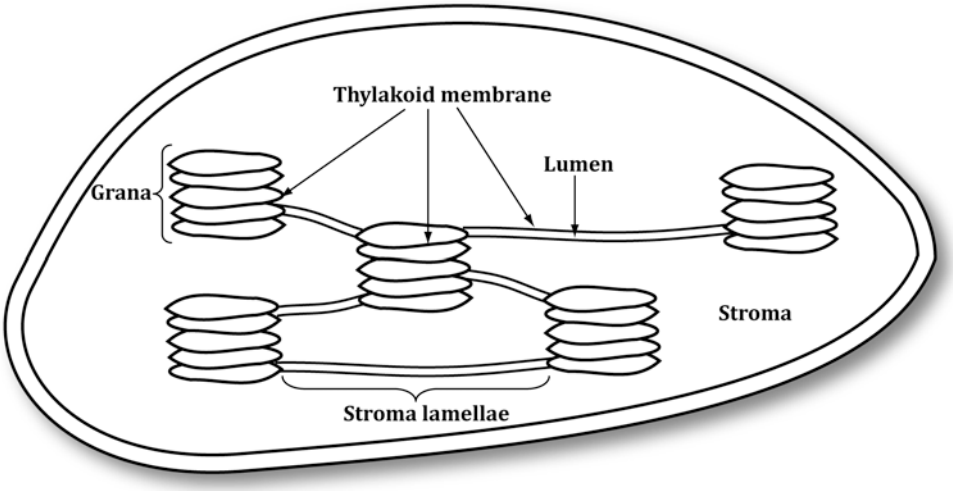

Figure 1. Schematic illustration of the chloroplast showing the appressed (grana) and the non-appressed (stroma lamellae) membrane regions of the thylakoid membrane as well as the soluble compartment enclosed by the thylakoids (lumen) and the surrounding stroma.

1.1. Oxygenic photosynthesis

The light-dependent photosynthetic reaction that takes place in the thylakoid membrane aims to produce NADPH and ATP, two high-energy rich compounds used in the Calvin-Benson-cycle to convert carbon dioxide into chemical energy in the form of sugar. This process is catalysed by four large protein complexes that reside within the thylakoid membrane: photosystem I (PSI), photosystem II (PSII), cytochrome b_6f and ATP-synthase (**Figure 2**). These complexes are not evenly distributed in the thylakoids. PSII is mainly found in the grana whereas PSI and ATP-synthase primarily localize to stroma lamellae. Cytochrome b_6f complexes are more evenly distributed but concentrate to grana and grana margins (Andersson and Anderson 1980; Albertsson et al. 1990). Associated with PSI and PSII are protein–pigment complexes (light-harvesting complexes; LHC) that enhance the light-harvesting by absorption of the incoming light and transfer of excitation energy to the reaction center of the photosystem. In PSII this event leads to oxidation of the reaction center Chla P680 which loses an electron that is transferred to the electron acceptor plastoquinone (PQ).

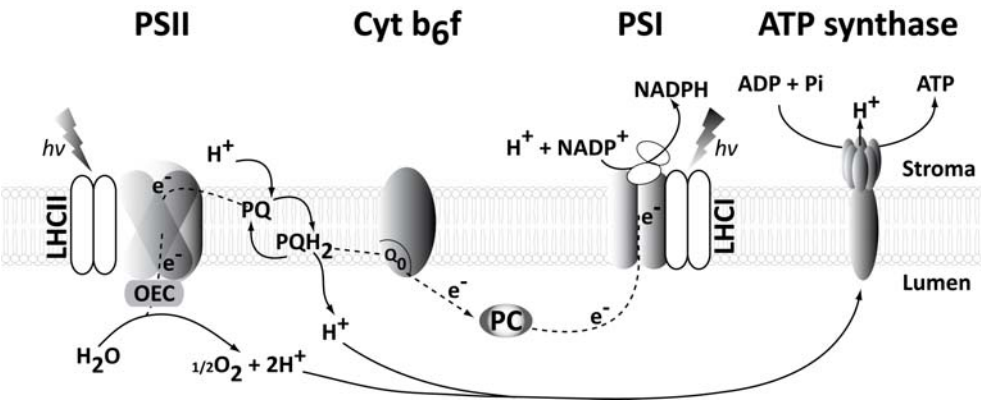

Figure 2. Linear electron flow and organization of major photosynthetic complexes in the thylakoid membrane. Dashed and solid lines represent the flow of electrons (e^-) and protons (H^+), respectively. The bolts (designated *hv*) illustrate light flashes.

Coincident with the oxidation of P680, electrons are withdrawn from water and transferred to the oxidized $P680^+$ by spitting of water into protons and oxygen, a process occurring on the lumenal side of the thylakoid membrane catalysed by the oxygen evolving complex (OEC). The reduced electron carrier PQ binds two protons from the stroma side of the membrane forming plastoquinol (PQH_2). PQH_2 associates with the cytochrome b_6f complex that mediates the transfer of electrons to a second electron carrier located within the lumen, plastocyanin (PC), and simultaneously releases two protons into the lumen. Plastocyanin, in turn, transfers electrons to the reaction center of PSI, P700, which has been oxidized via excitation by light absorbed by PSI and LHCI. Electrons obtained by oxidation of P700 are used by ferredoxin-$NADP^+$ oxidoreductase on the stromal side of the thylakoid membrane to form NADPH by reduction of $NADP^+$. Alternatively, these electrons can be used to reduce the cytochrome b_6f complex, resulting in the cyclic electron flow, as opposed to the linear flow described above. In both modes of electron transport, a proton gradient is built up across the membrane. It drives ATP-synthase to form ATP to be used in the Calvin-Benson-cycle as well is in a great number of cellular processes that require energy (Anderson and Andersson 1988; Nelson and Ben-Shem 2004; Barber 2007).

1.1.1. Acclimation mechanisms

Due to their stationary growing position, plants have to deal with changes in the surrounding environment on the spot where they are instead of moving to a more

favourable location. Plants are frequently exposed to changing conditions both on a minute (shading, cloudiness etc.), daily (light and dark) and seasonal (temperature, light, drought etc.) basis and have developed several acclimation mechanisms to adjust the photosynthetic machinery and optimize photosynthesis under the prevailing condition.
Although essential for photosynthesis, light can cause damage to the photosynthetic apparatus. Too much light leads to over-excitation of the photosystem since only a certain amount of excitation energy can be used for photosynthesis. To balance the over-excitation, excess energy can be dissipated as heat in a process known as non-photochemical quenching (NPQ) (recently reviewed in Ruban et al. 2012). Electron transport is a much slower process than energy transfer and under increasing light intensity the photosynthetic reaction centers gradually become saturated. Energy not utilized for photosynthesis is then accumulating and can cause damage to the photosynthetic machinery, in particular to PSII, leading to decreased photosynthetic efficiency. In NPQ, triggered by the increased transmembrane proton gradient, formation of zeaxanthin and monomerization of PsbS causes reorganization of LHCII-PSII supercomplexes and energy dissipation as heat protects the photosynthetic apparatus from damage (Ruban et al. 2012).

Light does not only vary in quantity but also in quality. The two photosystem reaction centers P680 and P700 have absorption maxima of slightly different wavelengths. In order not to waste any of the incoming light, light harvesting is balanced by shuffling of LHCII between PSII and PSI in a process called state transitions (Rochaix 2007; see section 1.4). State transitions are predominantly observed in low light, occur in the timescale of minutes and are an example of how the photosynthetic organism adapts to changes in the short term. In the long term acclimation process for the photosynthetic organism to adjust to the new prevailing condition, the photosynthetic machinery is modified in a more permanent way by changes in photosystem stoichiometry. This acclimation mechanism takes place in the timescale of hours to days and involves alterations in gene expression and synthesis of proteins associated with photosynthesis (Pfannschmidt et al. 1999; Dietzel et al. 2008). This event will be further described in section 1.5.

1.2. Immunophilins – a ubiquitous protein family

Reactions within the chloroplast stroma and the thylakoid membrane have been extensively studied for a long time. Up to, in that respect, recently, the thylakoid lumen was believed only to function as a proton sink for photosynthesis and to hold just a few proteins necessary for electron transport between the cytochrome b_6f complex and PSI. However, proteomic studies of this compartment have found many more proteins to be located in the thylakoid lumen (Kieselbach et al. 1998; Peltier et al. 2002; Schubert et al. 2002). Today, 80-200 proteins are found or predicted to reside in this compartment and it is reasonable to assume that many of them are involved in regulation and fine-tuning of photosynthesis. One characteristic of the thylakoid lumen proteome of Arabidopsis is the high number of proteins belonging to the immunophilin superfamily. Immunophilins are ubiquitous proteins expressed in both prokaryotic and eukaryotic organisms that were first discovered as targets of immunosuppressive drugs used to suppress the immune response after organ transplantations. Subsequently, they are classified according to which immunosuppressive drug they bind; cyclophilins bind Cyclosporin A (CsA) while FK506-binding proteins (FKBPs) bind FK506 (Handschumacher et al. 1984; Schreiber 1991).

In the same year as Handschumacher et al. (Handschumacher et al. 1984) reported the finding of Cyclophilin, Fischer et al. (Fischer et al. 1984) reported the finding of a protein able to catalyze the *cis-trans* isomerization of peptidyl-prolyl bonds. This enzyme was classified as a peptidyl-prolyl *cis-trans*-isomerase (PPIase) and was later realized to be identical to Cyclophilin (Fischer et al. 1989). Many proteins of the immunophilin superfamily, consisting of cyclophilins and FKBPs, together with a third protein family called parvulins, possess PPIase activity and are regarded as PPIases. Apart from other amino acids, proline (Pro) residues frequently occur in *cis* conformation in protein structures. The *cis* conformer represents a less energetically favourable state in the peptide backbone but for proline, with its unique imide peptide bond, the *cis* and *trans* isomers are closer in free energy and therefore occur more frequently in *cis* conformation. However, due to the energy barrier separating the two states, the intrinsic isomerisation of the prolyl bond is a slow process. PPIases are per definition able to catalyze this *cis-trans* isomerization of the bond preceding proline residues in the

polypeptide chain, a step regarded as a rate limiting step of protein (un)folding and a mechanism by which a molecule can switch between two functional states (Brandts et al. 1975; Galat 2003; Lu et al. 2007) (**Figure 3**).

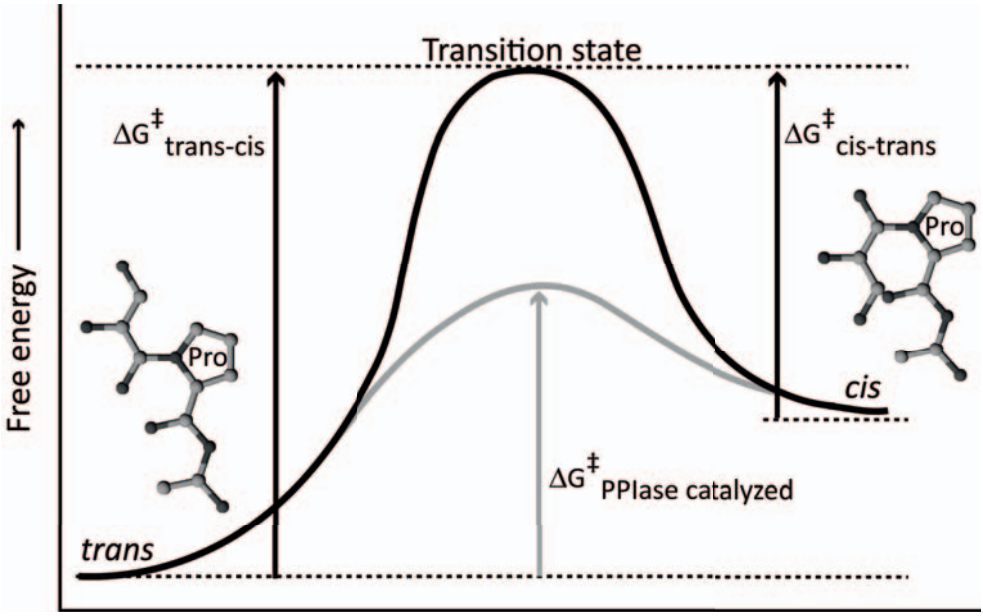

Figure 3. PPIases decrease the activation energy. In the presence of a PPIase, isomerization of the proline bond is accelerated by the decreased ΔG‡. Illustration was influenced by Lu et al. (Lu et al. 2007).

Much knowledge about the physiological significance of immunophilins comes from observations on their binding to immunosuppressive drugs. However, these drug-dependent functions do not tell the natural, 'true', physiological relevance of immunophilins in the absence of the immunosuppressive drug. Immunophilins are involved in a great variety of cellular processes: being parts of large chaperone complexes, protein folding, cell growth and proliferation, DNA binding, gene transcription, receptor stabilization/regulation, hormone signalling, protein trafficking, heat-shock responses and pathogen-host interactions (Freskgård et al. 1992; Göthel and Marahiel 1999; Galat 2003; Barik 2006; Bell et al. 2006; Geisler and Bailly 2007).

Although immunophilins are attributed with important functions, the importance of PPIase activity *per se* remains elusive. For example, when all PPIases in yeast were mutated they were found to be dispensable for the viability of the organism (Dolinski et al. 1997). Several immunophilins contain additional domains beside their PPIase domain

several of which are involved in protein-protein interactions to modulate protein functions (Galat 2003).

The genome of *Arabidopsis thaliana* encodes 52 immunophilins making this plant one of the most immunophilin-rich organisms characterized so far (He et al. 2004; Ahn et al. 2010). Some of these proteins have been found to be involved in gene regulation; FKBP53 is a histone chaperone repressing the expression of ribosomal genes and CYP71 was found to interact with histone H3 and key components in chromatin assembly and modification (Li et al. 2007; Li and Luan 2010; 2011). Similarly, CYP59 might control gene expression by interacting with a subunit of RNA polymerase II suggesting a role in RNA maturation (Gullerova et al. 2006). CYP40, FKBP42 and FKBP72 were all found to play important roles for plant development by regulating the transition from juvenile phase to adult phase, cell elongation, auxin signalling or cell proliferation (Vittorioso et al. 1998; Berardini et al. 2001; Geisler et al. 2003). FKBP73 was found as a co-chaperone of HSP90 while FKBP62 and FKBP65 were both shown to be involved in acquired thermotolerance (Meiri and Breiman 2009; Meiri et al. 2010; Fellerer et al. 2011).

1.2.1. Chloroplast immunophilins

Out of 52 potential immunophilins in Arabidopsis, 17 are predicted to reside within the chloroplast; one in the stroma and 16 inside the thylakoid lumen. The physiological significance for such a high number of immunophilins in the thylakoid lumen is not clear but is assumed to be associated with protein folding, biogenesis and maintenance of photosynthetic complexes (Lippuner et al. 1994; Peltier et al. 2002; Schubert et al. 2002; Romano et al. 2005). The first immunophilin to be found within the chloroplast was the stroma-located protein known as CYP20-3. CYP20-3 possesses PPIase activity but was found to be dispensable for plant growth under standard growth conditions. However, CYP20-3-deficient plants grown under high light were retarded in growth and displayed more rapid photodamage compared to wild type plants (Lippuner et al. 1994; Cai et al. 2008; Dominguez-Solis et al. 2008).

In recent years the potential functions for several lumenal immunophilins have started to emerge and many of them indeed seem to be involved in accumulation, regulation and maintenance of photosynthetic complexes. FKBP20-2-lacking plants have a stunted growth and are impaired in the accumulation of PSII supercomplexes (Lima et al. 2006). A yeast two-hybrid screen for interaction partners to wheat FKBP16-3 found two

proteins that are potential photosystem assembly factors and the same study also found wheat FKBP16-1 to interact with the PSI subunit PsaL essential for state transitions (Gollan et al. 2011). FKBP16-2 was found to stabilize the NAD(P)H dehydrogenase (NDH) complex and was proposed to be a subunit of a lumenal subcomplex of the NDH-PSI supercomplex (Peng et al. 2009). CYP20-2 was also proposed to be a subunit of the NDH-complex (Peng et al. 2009; Sirpiö et al. 2009) and have its expression regulated by light (Romano et al. 2004). In Arabidopsis, CYP20-2 is one of the two immunophilins with detectable PPIase activity in the thylakoid lumen. Mutants deficient in CYP20-2 do not show any change in phenotype and the PPIase activity in these mutants is indistinguishable from wild type activity due to a more active FKBP13 (Edvardsson et al. 2007). FKBP13 is the most active PPIase in the thylakoid lumen of Arabidopsis and its activity was found to be redox-dependent (Gopalan et al. 2004; Shapiguzov et al. 2006). Although being a lumenal protein, only the precursor form of Arabidopsis FKBP13 was found to interact with the Rieske protein of the cytochrome b_6f complex. FKBP13 was therefore proposed to sequester Rieske protein in the cytoplasm or in the chloroplast stroma to control its accumulation in the cytochrome b_6f complex (Gupta et al. 2002). However, a recent study did not detect such an interaction between the precursor of wheat FKBP13 and Rieske. Instead, only the mature form of the protein interacted with the Rieske protein indicating that the interaction between FKBP13 and Rieske occurs within the thylakoids and not in the chloroplast stroma (Gollan et al. 2011). CYP38 has a crucial role for plant growth. Plants deficient in CYP38 show a dramatic retardation of growth and are highly susceptible to photodamage. CYP38 was found to be necessary for correct assembly of PSII complexes (Fu et al. 2007; Sirpiö et al. 2008). It is a multidomain immunophilin with one phosphatase regulatory module apart from its cyclophilin domain. The spinach ortholog to CYP38, TLP40, was found to interact with a thylakoid membrane-bound protein phosphatase and to inhibit phosphatase activity (Fulgosi et al. 1998). Accordingly, mutants deficient in CYP38 have lower levels of phosphorylation of thylakoid proteins due to a higher dephosphorylation rate (Sirpiö et al. 2008).

1.3. Reversible protein phosphorylation in chloroplasts

A major mechanism for animals and plants to modify the properties of proteins on a posttranslational level is reversible protein phosphorylation.

Evidence for chloroplast protein phosphorylation was first reported by Bennett in 1977 who observed *in vitro* incorporation of ^{32}P-orthophosphate into a few chloroplast proteins (Bennett 1977). Recent mass spectrometric analyses of the chloroplast have revealed a complex phosphorylation network – the chloroplast phosphoproteome has expanded from a few proteins to around 200 (Sugiyama et al. 2008; Baginsky and Gruissem 2009; Lohrig et al. 2009; Reiland et al. 2009). Reversible protein phosphorylation requires enzymes that either add (kinases) or remove (phosphatases) phosphate groups on proteins.

Following the pioneer works by Bennett (Bennett 1977; 1980; Bennett et al. 1980), much focus has been on reversible phosphorylation of thylakoid membrane proteins (Allen 1992; Aro and Ohad 2003; Vener 2007). Several of the known phosphoproteins in thylakoid membranes of Arabidopsis are phosphorylated by two serine/threonine (Ser/Thr) protein kinases: STN7 and STN8 (Bellafiore et al. 2005; Bonardi et al. 2005; Vainonen et al. 2005). Both these kinases were discovered as they displayed sequence similarities to the thylakoid-associated protein kinases Stt7 and Stl1 in the green alga *Chlamydomonas reinhardtii* (Depege et al. 2003).

1.3.1. STN8-dependent phosphorylation

Phosphorylation of PSII subunits D1, D2, CP43 and PsbH is known to depend on the presence of STN8 which is regarded as the PSII core kinase (Bonardi et al. 2005; Vainonen et al. 2005). STN8 has also been found to phosphorylate other thylakoid proteins like the calcium-sensing receptor protein CaS and PGRL1-A, a protein probably involved in modulation of cyclic electron transfer (Vainonen et al. 2008; Reiland et al. 2011). Reversible PSII core phosphorylation controls the macroscopic structure of thylakoids and is believed to play an important physiological role in the turnover of light damaged PSII complexes (Baena-González et al. 1999; Fristedt et al. 2009b). Light of all intensities generates photo-oxidative damage to PSII and especially to the D1 protein. This would result in reduced PSII activity if damaged D1 was not replaced. Accordingly, phosphorylation of PSII subunits is increasing with increasing illumination. It has a key

role in the D1 repair cycle which involves the movement of phosphorylated PSII to the stroma-exposed thylakoids where the damaged D1 is replaced (Tyystjärvi and Aro 1996; Rintamäki et al. 1997; Baena-González et al. 1999). In plants with reduced PSII phosphorylation (e.g. the *stn8* and the *stn7xstn8* mutants), an enhanced grana size prevents lateral migration of light-damaged D1 to the non-appressed thylakoids which lowers its accessibility to proteases carrying out D1 turnover. Consequently, STN8-deficient plants accumulate more damaged PSII complexes under prolonged high light stress (Tikkanen et al. 2008; Fristedt et al. 2009b).

1.3.2. STN7-dependent phosphorylation

Contrary to STN8, the STN7 kinase is required for phosphorylation of proteins involved in light-harvesting. Subunits of LHCII, minor light-harvesting protein CP29 and the small LHCII interacting protein TSP9 are all known to undergo STN7-dependent phosphorylation (Bellafiore et al. 2005; Tikkanen et al. 2006; Fristedt et al. 2009a). In addition, as will be discussed in the results part, phosphorylation of the nucleoid-associated protein pTAC16 is also dependent on STN7 (Paper IV). Phosphorylation of LHCII and TSP9 is involved in state transitions (see below) while STN7-mediated phosphorylation of CP29 is needed for LHCII dissociation from PSII upon high light exposure (Bellafiore et al. 2005; Fristedt et al. 2009a; Fristedt and Vener 2011).

1.3.3. Regulation of STN7

Activation of LHCII phosphorylation mediated by STN7 depends on the redox state of the plastoquinone pool. Binding of plastoquinol to the Qo site of the cytochrome b_6f complex triggers STN7-dependent phosphorylation of LHCII while the reverse situation emerges when the plastoquinone pool is oxidized; plastoquinone is excluded from the Qo site leaving an inactive STN7 (Vener et al. 1995; Vener et al. 1997; Zito et al. 1999). However, the regulation of STN7 activity is complex and includes several different pathways besides the oxidation state of plastoquinone (Lemeille and Rochaix 2010; Puthiyaveetil 2011). Excessive light inhibits the STN7 kinase although the PQ pool is highly reduced. This inactivation of the kinase involves stroma components thought to be a ferredoxin-thioredoxin system (Rintamäki et al. 1997; Rintamäki et al. 2000). STN7 has two cysteins in the stromal parts of the protein as well as two cysteins in the lumenal part. Both sites are prone to thioredoxin-mediated reactions and two proposed models for the inhibition exist (Dietzel et al. 2008; Puthiyaveetil 2011). There are no

published data on the importance of the stroma-located cysteins. However, the absence of a disulfide bridge between the two lumenal cysteins makes the kinase inactive as found in mutagenesis studies of Stt7 (Lemeille et al. 2009). STN7 itself was found to be phosphorylated at several sites close to its C-terminal and results from site-directed mutagenesis of these phosphosites indicate that phosphorylation of STN7 may affect its turnover. However, phosphorylation of STN7 was not required for state transitions or LHCII phosphorylation (Reiland et al. 2009; Willig et al. 2011). Interestingly, STN8 is not required for phosphorylation of STN7 (Reiland et al. 2011; Paper IV).

1.3.4. Other chloroplast kinases

Although STN8 and STN7 are responsible for phosphorylation of several thylakoid phosphoproteins, some phosphorylation remains in the *stn7xstn8* double mutant. The residual light-independent phosphorylation of D2, PsbH and LHCII implicate another kinase in these events as well (Fristedt et al. 2009b). Evaluation of predicted chloroplast-localized kinases identified 15 kinases in the chloroplast, for most of which the substrates and functions are unknown (Bayer et al. 2012).

Except for STN7, a family of three thylakoid-associated kinases (TAKs) has also been found to phosphorylate LHCII (Snyders and Kohorn 1999). TAKs were found to co-localize with both cytochrome f and with LHCII. Antisense *tak1* plants are partly deficient in state transitions as well as in phosphorylation of both LHCII and PSII core proteins (Snyders and Kohorn 2001). Later studies did however only confirm the chloroplast localization for TAK1 but not for TAK2 and TAK3 (Schliebner et al. 2008).

The major phosphorylation activity in the chloroplast might be assigned to the chloroplast casein kinase 2 (CK2). Analysis of kinase target motifs in chloroplast phosphoproteins showed CK2 phosphorylation motifs to be the most common suggesting that CK2 functions as a central regulator of different chloroplast processes (Reiland et al. 2009). Among other chloroplast kinases of which the function has started to emerge is the chloroplast sensor kinase (CSK). CSK has been found to be an interaction partner of CK2 and to control transcription of chloroplast genes on the basis of the plastoquinone redox state (Puthiyaveetil et al. 2008; Puthiyaveetil et al. 2010).

1.3.5. Protein phosphatases

Except for kinases, reversible protein phosphorylation also requires the presence of phosphatases to counterbalance the action of kinases. Protein phosphatases are highly

conserved between different species and are grouped according to substrate specificity: Ser/Thr, Tyr and dual-specificity phosphatases. Ser/Thr phosphatases are built up by two families that lack sequence similarities – the protein phosphatase P (PPP) family and the protein phosphatase M (PPM) family (Barford 1996; Fauman and Saper 1996). Based on their substrate specificity, metal ion requirements for activity and sensitivity to different inhibitors, these can also be clustered into type 1 and type 2 phosphatases. Type 2 enzymes comprise PP2As and PP2Bs from the PPP family as well as PP2Cs from the PPM family. Enzymes within PP2A do not have any ion requirements while PP2Bs require Ca^{2+} and PP2Cs require Mg^{2+} or Mn^{2+} for their activity. Although no requirements for divalent cations, PP2As are built up by three subunits: a structural, a regulatory and a catalytic subunit. PP2Cs however, are monomeric enzymes (Cohen 1989; Barford 1996).

There are about 150 possible catalytic protein phosphatases encoded by the Arabidopsis genome. No members of the PP2B family are recognized while PP2Cs comprise the largest group with 80 potential enzymes. The Arabidopsis genome holds about four times as many genes for PP2Cs as the human genome suggesting a broader functional diversity of plant PP2Cs. Several plant PP2Cs have been found to act within the abscisic acid signalling network. Thus, PP2Cs appear to have important functions for tolerance to abiotic stresses in plants (Rodriguez 1998; Kerk et al. 2008; Xue et al. 2008).

1.3.6. Dephosphorylation of thylakoid proteins

Dephosphorylation of thylakoid proteins was found to occur in isolated thylakoid membranes implicating a thylakoid-bound protein phosphatase. The dephosphorylation rate was independent of light while the presence of only micromolar concentrations of Mg^{2+} resulted in rapid dephosphorylation of LHCII (Bennett 1980). Later investigations of *in vivo* dephosphorylation of thylakoid proteins found, however, several distinct dephosphorylation pathways. Indeed, LHCII dephosphorylation was found not to depend significantly on light. On the contrary, D1, D2 and CP43 showed light-stimulated dephosphorylation speaking in favour of at least two different protein phosphatases involved in dephosphorylation of thylakoid phosphoproteins (Elich et al. 1993; Rintamäki et al. 1996; Elich et al. 1997).

Intriguingly, dephosphorylation of D1 is both light-dependent and light-independent suggesting that more than one phosphatase has D1 as a substrate. Light-damaged D1 is not dephosphorylated in dark while dephosphorylation of D1 in fully functional PSII

centers is fast and independent of light (Rintamäki et al. 1996). As mentioned above, reversible phosphorylation of PSII core proteins is important for D1 turnover and dephosphorylation plays a critical role in this process. Replacement of D1 requires disassembly of the PSII monomer, a step mediated by dephosphorylation of CP43, D1 and D2. Dephosphorylation of D1 is also of importance for the turnover since the phosphorylated form of the protein is a poorer substrate for degradation (Ebbert and Godde 1996; Rintamäki et al. 1996; Baena-González et al. 1999).

The identities of the involved phosphatases for these events have remained ambiguous until very recently when both a phosphatase involved in dephosphorylation of LHCII, PPH1/TAP38, and a phosphatase involved in dephosphorylation of PSII core proteins, PBCP, were identified in Arabidopsis (Pribil et al. 2010; Shapiguzov et al. 2010; Paper II; Paper III; see major findings). However, several protein phosphatases with activities toward thylakoid proteins have been partially purified from different species in the past. A partially purified protein able to dephosphorylate thylakoid proteins was obtained by NaCl wash of wheat thylakoids. The activity of this extrinsic thylakoid membrane phosphatase was increased by addition of magnesium and manganese ions as well as by addition of reducing agents like 2-mercaptoethanol and DTT (Sun et al. 1989). Two protein phosphatases, one membrane-associated and one stroma-located, with activities toward LHCII phosphopeptides were identified in pea chloroplasts. Both were activated by DTT while they differed in their pH optimum, their response to magnesium and manganese ions and in their inhibition by molybdate ions (Hammer et al. 1995a; Hammer et al. 1995b; Hammer et al. 1997). Taken together, these results pointed out that dephosphorylation of LHCII seems to be mediated by a protein phosphatase of the PP2C family, as suggested by the Mg^{2+} and Mn^{2+} dependence of its activity (Carlberg and Andersson 1996). In addition, a PP2A-like protein phosphatase regulated by immunophilin TLP40 was isolated from spinach thylakoids (see section 1.2.1) (Vener et al. 1999). This integral membrane phosphatase was, in opposite to the other studied phosphatases, efficient in dephosphorylation of PSII phosphoproteins.

1.4. State transitions

One of the most studied events caused by reversible protein phosphorylation in the thylakoids is referred to as 'state transitions'. State transitions are a way for the photosynthetic apparatus to balance light harvesting between PSI and PSII in order to adjust to changes in light quantity/quality within a few minutes (Allen 1992; Allen and Forsberg 2001; Rochaix 2007). This phenomenon was first observed in red alga (Murata 1969) and green alga (Bonaventura and Myers 1969) already in 1969. Later it was found that this redistribution of energy between the two photosystems was dependent on phosphorylation and the PQ redox state (Bennett et al. 1980; Allen et al. 1981).

PSI and PSII preferentially absorb light of slightly different wavelengths and in the natural environment the incoming light can be fluctuating in both quantity and quality over time. In order not to waste any of the available light as a consequence of one photosystem being held back by the other, a mobile pool of LHCII shuttles between the two photosystems to aid the underexcited one and to optimize light harvesting (Allen and Forsberg 2001). This event is accompanied by a large structural rearrangement of thylakoid membranes (Chuartzman et al. 2008). Light preferentially absorbed by PSII will cause a migration of a mobile LHCII pool to PSI increasing the ability of the latter to harvest light (state 2). On the contrary, preferential excitation of PSI will lead to LHCII relocation to PSII (state 1).

A prerequisite for this rearrangement of LHCII is the redox-controlled LHCII phosphorylation that is regulated by changes in the oxidation state of plastoquinone (Allen et al. 1981; Bellafiore et al. 2005). Upon state 2-favouring light, the reduced plastoquinone pool will activate the LHCII kinase, STN7, by binding of plastoquinol to the Qo site of cytochrome b_6f complex (Zito et al. 1999). An activated STN7 leads to phosphorylation of LHCII and its subsequent migration to PSI. Conversely, STN7-mediated phosphorylation of LHCII is inactivated by an oxidized plastoquinone pool in the dark or when PSI is favoured, leading to dephosphorylation of LHCII by the recently identified protein phosphatase PPH1/TAP38 and to LHCII reassociation with PSII (Rochaix 2007; Pribil et al. 2010; Shapiguzov et al. 2010; Paper II) (**Figure 4**).

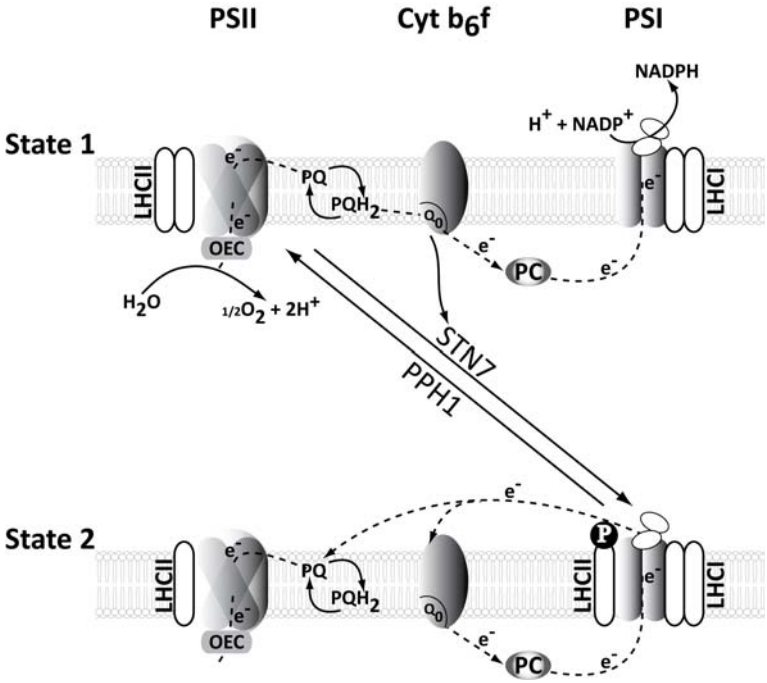

Figure 4. The classical view of state transitions. A transition from State 1 to State 2 is initiated by a more reduced PQ-pool upon excitation of PSII. Binding of PQH_2 to the Qo site of the cytochrome b_6f complex leads to phosphorylation of LHCII by activation of the protein kinase STN7. Phosphorylated LHCII (designated 'P') dissociates from PSII and migrates to PSI to aid its light harvesting. In green algae, transition from state 1 to state 2 is accompanied by a shift from linear electron flow to cyclic electron flow. Upon inactivation of STN7 (i.e. oxidation of the PQ-pool), dephosphorylation of LHCII mediated by the protein phosphatase PPH1 causes LHCII reassociation with PSII (state 1). Illustration was influenced by J. D. Rochaix (Rochaix 2011).

State transitions are more pronounced in the green alga *Chlamydomonas reinhardtii* where 80% of the LHCII represents the mobile LHCII pool in contrast to plants where only 20% are found to relocate during state transitions (Allen 1992; Delosme et al. 1996). In *Chlamydomonas*, state transitions allow the photosynthetic organism to switch between linear and cyclic electron flow depending on its metabolic state (Finazzi and Forti 2004). In plants however, such a switch has not been found and the physiological relevance of state transitions in land plants is debated. In fact, only minor impairments in plant fitness were found for field-grown *stn7* mutants and plants deficient in state transitions were even found to be able to compensate for their defect (Lunde et al. 2003; Frenkel et al. 2007). However, STN7-lacking plants display a severe retardation of growth when subjected to fluctuating light conditions (Bräutigam et al. 2009; Tikkanen

et al. 2010) and the ability to perform state transitions was found critical for photosynthesis in greenhouse-grown plants when the linear thylakoid electron transport was perturbed (Pesaresi et al. 2009).

1.5. Redox control of gene expression

Except from being necessary for short term regulation of photosynthesis, the redox state of the PQ pool and protein phosphorylation also play a role in the adaptation of the photosynthetic apparatus in the long term acclimation (LTR) to changes in the surrounding environment. The rate of transcription of genes for the two major subunits of PSI, *psaAB*, as well as for the gene coding for D1, *psbA*, were found to depend on the redox state of PQ. Mustard plants exposed to light favouring PSII or to inhibitors of electron transfer from PQH_2 increased the rate of *psaAB* transcription while a decrease in transcription rate was observed when plants encountered PSI-favouring light or DCMU treatment causing an oxidized PQ pool. In contrast, in plants subjected to PSII or PSI light, the transcription rate for *psbA* was repressed and promoted, respectively (Pfannschmidt et al. 1999). However, the *psbA* gene does not seem to be under redox control in Arabidopsis and pea (Tullberg et al. 2000; Pesaresi et al. 2009). Similar to state transitions, this adjustment of photosystem stoichiometry counteracts the imbalance formed when one photosystem is held back by the other but in a more permanent way compared to state transitions. Except involving changes in plastid and nuclear gene expression, LTR also induce distinct adjustments of plant metabolism (Bräutigam et al. 2009).

psaAB and *psbA* represent genes encoded by the chloroplasts own genome. Transcription of chloroplast-encoded photosynthetic-associated genes is primary mediated by the plastid-encoded RNA polymerase (PEP) together with a number of associated nuclear-encoded factors (Hajdukiewicz et al. 1997; Liere and Maliga 2001). PEP-associated proteins (kinases, sigma factors, DNA/RNA-binding proteins, proteins involved in translation etc.) are believed to have regulatory functions in transcription and a redox control of expression of key photosynthetic genes provides a rapid and direct pathway by which the state of photosynthesis modulates its stoichiometry to meet the prevailing condition (Pfannschmidt and Liere 2005; Pfalz et al. 2006). The signal transduction from the redox state of PQ to gene expression is currently believed

to include a phosphorylation cascade (Pesaresi et al. 2009; Steiner et al. 2009; Puthiyaveetil et al. 2010). For example, the kinase CSK controls transcription of chloroplast genes on the basis of the redox state of PQ, thus providing a link between photosynthesis and gene expression (Puthiyaveetil et al. 2008). CSK has also been found to be an interaction partner of the PEP-interacting kinase CK2 in a yeast two-hybrid system. CK2 is known to regulate gene transcription via phosphorylation of sigma-like transcription factors and to be a subject to phosphorylation itself *in vitro*. The kinase activity of CK2 was dependent on its phosphorylation state which suggests a possible phosphorylation signalling cascade controlling chloroplast gene transcription. Consequently, CSK was suggested to phosphorylate CK2 (Baginsky et al. 1997; Baginsky et al. 1999; Ogrzewalla et al. 2002; Puthiyaveetil et al. 2010; Schweer et al. 2010). Recently, evidence for a role for STN7 in these LTR events has been presented (Bonardi et al. 2005; Pesaresi et al. 2009). Interestingly, also the expression of some nucleus-encoded genes seems to depend on the presence of STN7 although only a few of them appear to be directly involved in photosynthesis. Regulation of the abundance of nucleus-encoded photosynthesis-related proteins might instead occur on a post-transcriptional level mediated by a STN7-dependent signalling cascade. One candidate proposed for providing a link between STN7 dependent phosphorylation and gene transcription is the above-mentioned kinase CSK (Pesaresi et al. 2009).

2. METHODOLOGIES

2.1. *Arabidopsis thaliana* as a model organism

A milestone for plant research was set in the year 2000 as a result of the complete sequencing of the *Arabidopsis thaliana* genome (The Arabidopsis Genome Initiative 2000). Arabidopsis is a small flowering plant in the mustard family with several advantages for research in plant biology and the analysis of complex organisms in general. Its relatively small sequenced genome, its short generation time, the large amount of offspring produced and the straightforward way to cultivate it have made Arabidopsis the plant of choice for many plant researchers (Meinke et al. 1998). The possibility to modify the Arabidopsis genome by *Agrobacterium tumefaciens*-mediated incorporation of T-DNA segments has dramatically increased our understanding of the

function for many individual genes/proteins in recent years. A large number of T-DNA insertion mutants are today available via stock centers.

In my work (Paper I-IV), I took advantage of the availability of such mutants to study basic cell biological regulation mechanisms.

2.2. PPIase activity measurements

To measure the PPIase activity in samples from the chloroplast thylakoid lumen, the chymotrypsin-coupled enzymatic assay described by Kofron et al. (Kofron et al. 1991) was used. Lumenal proteins were obtained after rupture of isolated thylakoids membranes by Yeda press treatment and subsequent ultracentrifugation to remove residual membrane particles (Kieselbach et al. 1998). After determination of protein concentration (Bradford 1976), various amounts of lumenal proteins were mixed with chymotrypsin and two different colorimetric peptide substrates, Suc-Ala-Ala-Pro-Phe- *p*-nitroanilide or Suc-Ala-Leu-Pro-Phe- *p*-nitroanilide (A- and L-peptides, respectively). The use of two different peptide substrates was to ensure identification of active isomerases from both cyclophilin and FKBP families since cyclophilins and FKBPs have different substrate specificities. FKBPs are more active toward substrates with a bulky amino acid prior to the proline residue (e.g. the L-peptide) while isomerization by cyclophilins shows little dependence on the residue prior to proline (Harrison and Stein 1990). The conjugated chromophore, *p*-nitroanilide, constitute the essence of the PPIase assay. Free *p*-nitroaniline has a yellow colour that can be monitored at 390 nm in a spectrophotometer. However, free *p*-nitroaniline will only be present in the reaction mixture upon release by chymotrypsin cleavage of the bond between *p*-nitroanilide and Phe. The peptide substrates can adopt both *cis* and *trans* conformation but chymotrypsin is only able to hydrolyse the bond between *p*-nitroanilide and Phe when the peptide is in the *trans* conformation. As a consequence, the *cis* to *trans* transition run faster in the presence of a PPIase compared to the spontaneous reaction resulting in a more rapid increase in absorbance at 390 nm following the release of *p*-nitroaniline (**Figure 5**).

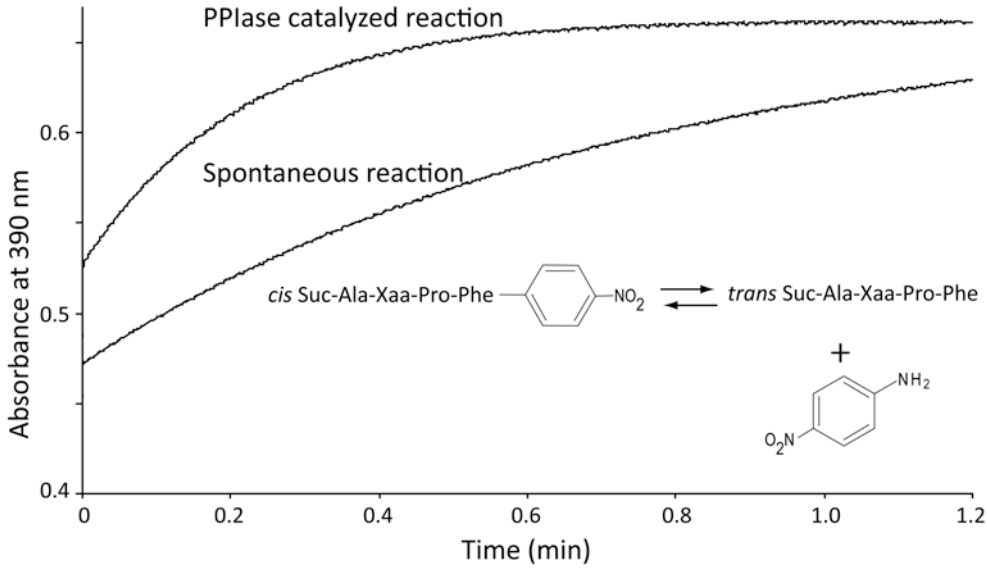

Figure 5. The PPIase activity assay. Typical traces for measurements of PPIase activity in a spontaneous and a PPIase-catalyzed reaction are shown. Cleavage of the bond between Phe and *p*-nitroanilide when the peptide substrate is in *trans* conformation is mediated by chymotrypsin. A concomitant increase in absorbance at 390 nm is observed upon release of *p*-nitroaniline.

2.3. Mass spectrometry

The introduction of mass spectrometry (MS) into the field of proteomics started with the development of two techniques able to gently disperse proteins and other macromolecules into the gas phase, electrospray ionization (ESI) and matrix-assisted laser desorption-ionization (MALDI) (Tanaka et al. 1988; Fenn et al. 1989). The development of these techniques resulted in a shared Nobel Prize in chemistry 2002. Today, mass spectrometry represents an invaluable tool to study the ever-changing proteome. MS is commonly used in proteomics for protein identification and can be used for large scale studies of entire proteomes. Tandem mass spectrometry (MS/MS) is used for peptide sequence determination by fragmentation of peptides (**Figure 6**).

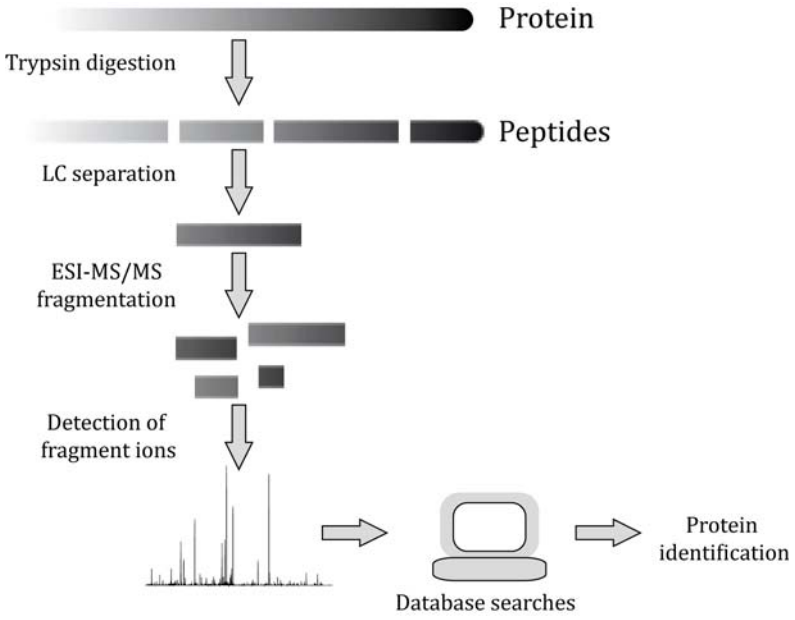

Figure 6. Protein identification using LC-ESI-MS/MS. A typical LC-MS/MS procedure involves proteolysis of proteins into peptides using a protease. Peptides are loaded on a reverse-phase LC-column and separated according to hydrophobicity. Subsequently, eluted peptides undergo vaporization and are separated according to their mass–to–charge ratio in the mass spectrometer. Selected peptide ions are fragmented and yield a fragmentation spectrum that is matched to known peptide sequences in a database.

Here, a selected peptide will collide with an inert gas and dissociate into fragments (collision induced fragmentation; CID) producing the resulting MS/MS spectrum. Based on these fragment ions and the knowledge of the mass for each residue, the amino acid sequence of the sequenced peptide can be determined (Mann et al. 2001). MS is also widely used to study covalent posttranslational protein modifications, like phosphorylations. In MS, the mass–to–charge ratio of a molecule is measured yielding a precise mass of the analysed compound. A modification will alter the mass of the residue on which it is positioned making it possible to determine the exact site of the modification in the protein sequence (Mann and Jensen 2003).

Fragmentation of peptides by CID is a harsh fragmentation technique that in some cases results in the loss of post translational modifications. A new, softer, fragmentation technique, electron transfer dissociation (ETD), has been developed to preserve posttranslational modifications during the MS analysis thus improving the use of MS in

the analyses of these modifications (Syka et al. 2004). Due to sample complexity, it is often necessary to make some kind of sample separation before the MS analysis. Many mass spectrometers can be conjugated with a liquid chromatography (LC) system that separates peptides by hydrophobicity before they are subjected to ionization and transferred to the mass spectrometer for analysis.

In my work (Paper I-IV) I have been using an ESI instrument able to fragment peptides both by CID and ETD (HCTultra PTM Discovery System, Bruker Daltonics). The mass spectrometer was equipped with a LC-system and the MS-based results in Paper II-IV are obtained by the use of a LC-system using flow rates in nanoliter-scale which further improve the separation of peptides and sensitiveness of the MS analysis.

2.3.1. Enrichment and quantification of phosphorylated peptides

Although protein phosphorylation is a very common post-translational modification, its low abundance makes mass spectrometric identification and quantification a challenge (Mann and Jensen 2003). To circumvent the problem with low abundance, there are ways for enrichment of phosphorylated peptides from complex biological samples prior to MS (Dunn et al. 2009). One common used method is enrichment by immobilized metal-affinity chromatography (IMAC). IMAC takes advantage of the fact that the phosphate covalently bound to the peptide is negatively charged and can interact with positively charged metal ions acting as a stationary phase (Andersson and Porath 1986). Several different metal ions can be employed for specific enrichment of phosphopeptides, but Fe(III) has been found suitable for enrichment of phosphorylated peptides from photosynthetic membranes (Vener et al. 2001). However, despite reducing the sample complexity, IMAC suffers from extensive non-specific binding of peptides that contain negatively charged amino acid residues (i.e. aspartic and glutamic acid). To overcome this problem, carboxyl groups present in the peptides can be converted into methyl esters by esterification (Ficarro et al. 2002). The extra step of esterification prior to enrichment also allows for differential labelling of samples using stable isotopes. This approach was used to quantify differences in protein phosphorylation of thylakoid proteins between wild type and mutants in Paper II and III.

Peptides from photosynthetic membrane proteins were obtained via trypsin treatment of isolated thylakoids, an approach referred to as vectorial proteomics (Vener et al.

2001; Vener and Strålfors 2005). Here, only surface exposed tryptic peptides will be collected from the thylakoid membranes, reducing the sample complexity considerably. Due to the hydrophilic nature of the phosphorylation event, phosphorylated fragments reside in the extrinsic parts of the membrane and are prone to release upon trypsin treatment.

Before further enrichment of phosphopeptides by IMAC, esterification with stable isotopes was performed. The tryptic peptides from wild type thylakoids were esterified with d0-methanol (modification of each carboxyl group gives a peptide mass increment of 14 Da), whereas peptides from the mutant were esterified with d3-methanol (peptide mass increment of 17 Da per modified carboxyl group). The reciprocal experiment (mutant peptides esterified with d0-methanol; wild type peptides with d3-methanol) was also performed as an internal control. Peptides were then mixed 1:1 prior to enrichment by IMAC and subsequent analysis using nLC-MS/MS.

The fact that peptides originated from the wild type and peptides originated from the mutant had slightly different masses, following the esterification with 'light' or 'heavy' methanol, made it possible to distinguish between them in the mass spectrometer. Quantitative comparison of intensities for light and heavy isotope–labeled phosphopeptide pairs was then performed to reveal differences in phosphorylation between the mutant and wild type proteins.

3. AIMS OF THE RESEARCH

The general aim of this thesis work has been to study ubiquitous cell biological regulatory mechanisms with focus on reversible modifications of chloroplast proteins in regulation of photosynthesis.

The more specific aims for the discussed projects were as follows:

Paper I: To examine the importance of PPIase activity in the thylakoid lumen of Arabidopsis by isolation and characterization of knockout mutants deficient in lumenal PPIase activity.

Paper II & III: To identify long sought protein phosphatases involved in dephosphorylation of thylakoid proteins and to characterize knockout mutants deficient in those enzymes.

Paper IV: To make a comparative chloroplast phosphoproteomic analysis of thylakoid kinase knockout mutants in order to find novel substrates of these enzymes involved in regulation of photosynthesis.

4. MAJOR FINDINGS

4.1. Characterization of Arabidopsis mutants deficient in lumenal PPIase activity (Paper I)

Despite the large number of immunophilins in the thylakoid lumen, only two were found to hold most of the conserved residues required for PPIase activity (Edvardsson et al. 2007). Subsequently, measurements of PPIase activity in isolated thylakoid lumen samples from Arabidopsis found only FKBP13 and CYP20-2 to possess PPIase activity (Shapiguzov et al. 2006). In the absence of the latter, a more active FKBP13 was compensating for the loss of activity leaving the net PPIase activity in the mutant lumen unaltered (Edvardsson et al. 2007). The finding that another PPIase was compensating for the loss of PPIase activity in the *cyp20-2* mutant suggested that a constant PPIase activity is important for the plant since there seems to be a backup system keeping the activity at a constant level. In Paper I we aimed to answer the question about the importance of PPIase activity in the thylakoid lumen of Arabidopsis by isolation and characterization of both a single knockout mutant of the FKBP13 gene and a double mutant deficient in both CYP20-2 and FKBP13.

4.1.1. PPIase activity depletion in the fkbp13 mutant

In sharp contrast to the *cyp20-2* mutant, plants lacking FKBP13 showed a dramatic decrease in lumenal PPIase activity (**Figure 7**). Residual PPIase activity was only found in fractions containing CYP20-2 and CYP20-3 after fast protein liquid chromatography fractionation of *fkbp13* lumen. As written above, CYP20-3 is a PPIase located in the chloroplast stroma present in lumen preparations as a contaminant. Regardless of the decrease of PPIase activity in the thylakoid lumen of *fkbp13*, mutant plants grew as good as wild type plants in normal growth conditions as well as under high light and cold stress. As PPIases are believed to act as protein-folding catalysts, we also characterized the *fkbp13* mutant at the protein level using immunoblotting with various antibodies toward thylakoid proteins and quantitative mass spectrometry using stable isotope-coded protein labelling (Figure 4 and Table 1 in Paper I). Neither of the approaches revealed any difference between wild type and FKPB13-depleted plants.

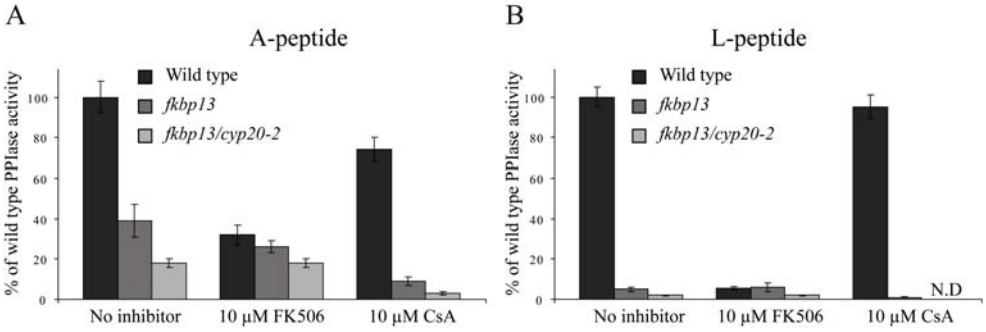

Figure 7. PPIase activity in isolated thylakoid lumen samples from wild type, *fkbp13* and *fkbp13/cyp20-2* plants assayed with A- and L- peptide substrates (A and B, respectively). Activities measured with or without inhibitors of PPIase activity are expressed as percentage of non-inhibited wild type. No activity was determined in samples from *fkbp13/cyp20-2* after inhibition of CsA when assayed with the L-peptide substrate.

4.1.2. Characterization of the fkbp13/cyp20-2 mutant depleted in lumenal PPIase activity

The generation of a double mutant lacking both active lumenal PPIases was obtained by crossing of the *fkbp13* and *cyp20-2* single mutants. This mutant would further address the question about the importance of PPIase activity in the thylakoid lumen. Similar to what was found for the corresponding single mutants, *fkbp13/cyp20-2* double mutant plants grew as good as the wild type. When exposed to stress conditions like low temperature or high light, the resulting phenotype of the double mutant resembled that of the wild type. Repetitive 2D gel separation of lumenal samples did not reveal significant reproducible changes in protein pattern between mutant and wild type except for the absence of FKBP13 and CYP20-2 in the mutant. The 2D gel experiment also showed the presence of the stromal contaminant CYP20-3 in these lumen samples. Accordingly, measurements of the PPIase activity in the lumen samples from the mutant found some residual PPIase activity (**Figure 7**). As with the *fkbp13* single mutant, residual activity was completely abolished after treatment with CsA indicating PPIase activity produced by a cyclophilin in line with the observation that CYP20-3 is present in the sample. Contribution to the measured activity by CYP38 was ruled out by measurements of PPIase activity in samples highly enriched in CYP38 which did not show any detectable activity.

4.1.3. FKBP13 and Rieske protein

Immunoblotting with antibodies against various thylakoid proteins in samples from wild type and the *fkbp13/cyp20-2* mutant did not reveal any significant differences in amount of analysed proteins similar to what was found for the *fkbp13* single mutant. Interestingly, immunoblotting analysis with antibody against the Rieske protein of the cytochrome b_6f complex did not show any change in accumulation of Rieske in the thylakoid membrane between wild type, *fkbp13* and *fkbp13/cyp20-2* (**Figure 8**). In an *in vitro* study, the precursor of FKBP13 was found to interact with Rieske (Gupta et al. 2002). It was proposed that FKBP13 would act as an anchor chaperone that sequestered Rieske in the cytoplasm or in the stroma to control its accumulation in the thylakoid membrane. Following that theory, it was suggested that more Rieske would be targeted to the thylakoids in the absence of FKBP13. However, our results from immunoblotting with Rieske antibody did not support such a role for FKBP13 since equal amounts of Rieske were detected in the thylakoids from wild type and the *fkbp13* and *fkbp13/cyp20-2* mutants.

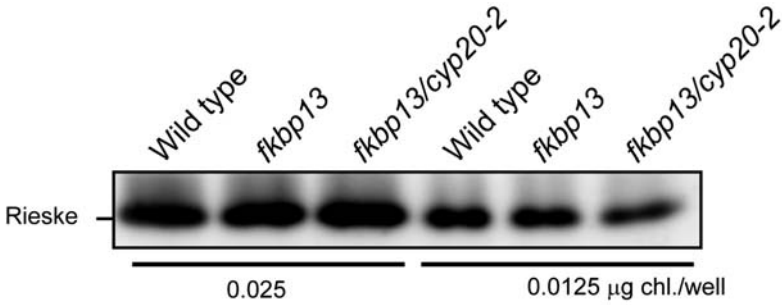

Figure 8. Immunoblot analysis of Rieske levels in thylakoids from wild type, *fkbp13* and *fkbp13/cyp20-2* mutants using antibody against Rieske. Samples were adjusted to the same chlorophyll content, as indicated.

In a recent study, the ortholog to FKBP13 in wheat was also found to interact with Rieske in a yeast two-hybrid assay. However, in sharp contrast to what was found by Gupta et al. (Gupta et al. 2002), the precursor of FKBP13 was not found to interact with Rieske. Instead, only the mature form of FKBP13 was found to interact with Rieske pointing towards an interaction between the two proteins within the thylakoids and not in the chloroplast stroma (Gollan et al. 2011). The interaction between FKBP13 and Rieske is indeed interesting since Rieske is suggested to have an important role for STN7 activity during state transitions (Lemeille et al. 2009) and the PPIase activity of FKBP13

is redox-regulated (Gopalan et al. 2004; Shapiguzov et al. 2006). Based on this, it is possible to speculate about a role for FKBP13 in the regulation of state transitions. Similarly, wheat FKBP16-1 interacts with the PSI subunit PsaL, required for state transitions, *in vitro* (Lunde et al. 2000; Gollan et al. 2011). However, when we analysed the ability of FKBP13-deficient plants to perform state transitions, no differences to wild type were observed (Shapiguzov, Ingelsson, Goldschmidt-Clermont and Vener, unpublished results).

4.1.4. PPIase activity is a dispensable function of immunophilins in the thylakoid lumen

From the results obtained in Paper I it is evident that lumenal PPIase activity is dispensable for plant survival. In contrast to FKBP13 and CYP20-2-lacking plants, mutant plants deficient in lumenal immunophilins CYP38 and FKBP20-2 display severe phenotypes (Lima et al. 2006; Fu et al. 2007; Sirpiö et al. 2008). No PPIase activity is recognized for CYP38 while recombinant FKBP20-2, although only having 2 out of 5 amino acids required for catalysis, were found to possess a very low PPIase activity (1/500 of that of FKBP13) (Lima et al. 2006). Recombinant proteins of wheat orthologs to Arabidopsis lumenal immunophilins FKBP16-1 and FKBP16-3 did not show PPIase activity (Gollan et al. 2011) and there are several examples of cyclophilins and FKBPs that lost their PPIase activity but still exhibit important functions. Except for CYP38 and FKBP20-2, the Arabidopsis protein FKBP42, having fundamental roles for the plant in cell elongation and auxin signalling, does not possess PPIase activity (Geisler et al. 2003). Wheat FKBP73 forms a complex with HSP90 but its chaperone activity was independent of its PPIase activity (Kurek et al. 2002) and similar observations were made for FKBP53 in Arabidopsis. It was found to interact with histone H3 through its acidic domains but the PPIase domain was dispensable for both histone chaperone activity and histone binding (Li and Luan 2010). Also, although having fundamental role for plant development, no evidence for the importance of PPIase activity were presented for other studied Arabidopsis immunophilins (Vittorioso et al. 1998; Berardini et al. 2001). Instead of relying on the PPIase activity, many of the multidomain-containing immunophilins modulate client proteins via direct protein-protein interactions. Conserved PPIase activity was proposed for FKBPs associated with stress responses to fulfil their chaperone function while others have decreased or lost the ability to possess

PPIase activity (Geisler and Bailly 2007). In regard to stress, CYP20-2 was found to have its expression regulated by light (Romano et al. 2004) and to be strongly upregulated under salt and desiccation stress treatment of rice plants (Ahn et al. 2010). The expression of the rice ortholog of FKBP13 did not, however, respond to salt and desiccation stress.

In conclusion, it is interesting to notice that among the four lumenal immunophilins for which T-DNA mutants have been characterized, two immunophilins possessing no or very low PPIase activity (CYP38 and FKBP20-2) were found to be crucial for plant growth (Lima et al. 2006; Fu et al. 2007; Sirpiö et al. 2008) while the mutants deficient in the two immunophilins contributing to all measureable lumenal PPIase activity (FKBP13 and CYP20-2) (Edvardsson et al. 2007; Ingelsson et al. 2009; Paper I) did not differ from wild type plants. The findings presented in Paper I postulate that the functions of immunophilins in the thylakoid lumen of Arabidopsis are not related to their ability to possess PPIase activity and they should be investigated beyond this enzymatic activity.

4.2. Identification of two protein phosphatases involved in dephosphorylation of thylakoid proteins (Paper II & III)

The first evidence for reversible protein phosphorylation in the thylakoid membrane was reported by Bennett in 1977 and three years later Bennett published evidence for a thylakoid-bound protein phosphatase which favoured substrate was LHCII (Bennett 1977; 1980). While protein kinases responsible for phosphorylation of thylakoid proteins have been identified previously (Snyders and Kohorn 1999; Bellafiore et al. 2005; Bonardi et al. 2005; Vainonen et al. 2005) the nature of the corresponding phosphatases have remained unknown. However, data from published mass spectrometric analysis of chloroplasts (Zybailov et al. 2008) and a genetic screen for chloroplast phosphatases led us to characterization of two protein phosphatases of the PP2C family, PPH1 and PBCP (Paper II and Paper III, respectively).

4.2.1. Characterization of the pph1 mutant deficient in LHCII dephosphorylation (Paper II)

An Arabidopsis mutant with a T-DNA insert in the gene coding for PPH1 showed higher levels of remaining LHCII phosphorylation in dark and in far red light compared to the wild type when analysed using immunoblotting with phosphothreonine antibodies.

Phosphorylation of major phosphoproteins of photosystem II was however similar indicating that PPH1 is involved only in dephosphorylation of LHCII (**Figure 9**; Figure 1 in Paper II).

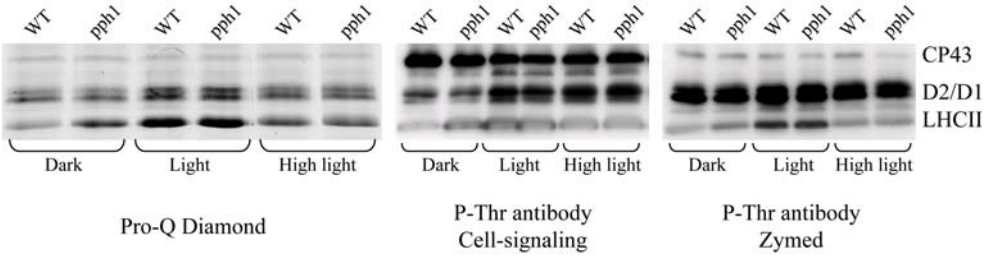

Figure 9. Analysis of thylakoid protein phosphorylation in wild type and *pph1* plants using phosphoprotein staining with Pro-Q Diamond and immunoblotting with two different phosphothreonine antibodies, as indicated. High light samples originate from plants that were light-adapted for 3 h and moved to high light conditions for 3 h prior to isolation of thylakoids.

The LHCII antenna is build up by several isoforms of Lhcb proteins of which many can undergo phosphorylation (Jansson 1999; Vener et al. 2001). To answer the question which Lhcb isoforms were not dephosphorylated in the mutant, a quantitative mass spectrometric approach with differential stable isotope labelling of peptides was used. This way two phosphorylated peptides from LHCII proteins were found to differ significantly between wild type and *pph1* while the levels of phosphorylation for the photosystem II core proteins D1 and D2 were similar in both genotypes after exposure to far-red light (**Figure 10**; Table 1 in Paper II). It should be noted however that these two LHCII peptides can originate from seven different Lhcb gene products due to high sequence similarity.

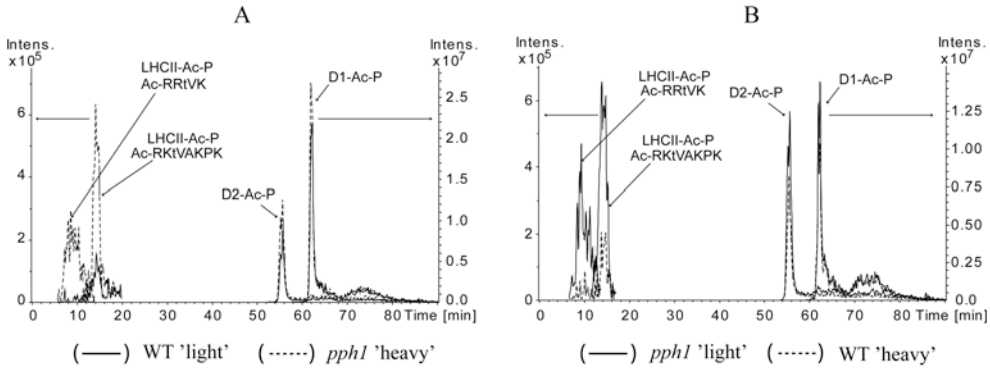

Figure 10. MS analysis of thylakoid protein dephosphorylation using IMAC. (A) Extracted ion chromatograms of phosphorylated peptides from the *pph1* mutant labeled with heavy isotope (dashed line) and wild type labeled with light isotope (solid line) analyzed by nLC-MS. Peaks corresponding to two N-terminal peptides from LHCII and to two N-terminal peptides from PSII (D1 and D2) are marked. (B) Extracted ion chromatograms from the reciprocal labeling experiment where peptides from the *pph1* mutant were labeled with light isotope (solid line) and peptides from wild type were labeled with heavy isotope (dashed line).

PPH1 meets the observation that the LHCII phosphatase requires Mg^{2+} for dephosphorylation of LHCII as this characteristic is specific for protein phosphatases of the PP2C family of which PPH1 is a member (Bennett 1980; Cohen 1989; Carlberg and Andersson 1996; Kerk et al. 2002). Investigation of the subcellular localization of PPH1 found it to accumulate in the stroma lamellae membranes of Arabidopsis thylakoid membranes (Figure 3 in Paper II). The location of PPH1 in the stroma lamellae is reasonable since this is the site to where phosphorylated LHCII migrates during state transitions and efficient dephosphorylation of LHCII occur (Andersson et al. 1982; Larsson et al. 1983; Carlberg and Andersson 1996). It was noted that induction of LHCII dephosphorylation was less effective in Arabidopsis mutants lacking the photosystem I subunit H (Lunde et al. 2000). PSI-H is responsible for the docking of LHCII to PSI during state transitions and the mutant is locked in state 1. The less effective dephosphorylation of LHCII might be due to the lack of substrate availability for PPH1 in the stroma lamellae when phosphorylated LHCII remains at PSII.

4.2.2. PPH1 in state transitions

Reversible phosphorylation of LHCII is a prerequisite for state transitions (Rochaix 2007). Measurements of state transitions in *pph1* revealed that this mutant is impaired in the relocalization of LHCII and that the relative PSI fluorescence was higher in the

mutant compared to wild type (Figure 4 in Paper II). This suggests that the mobile LHCII antenna is retained at photosystem I (state 2) in the mutant even after a light shift promoting state 1. In contrast, the *stn7* kinase mutant lacking the state transitions kinase STN7 is locked in state 1 since no phosphorylation of LHCII occur (Bellafiore et al. 2005). PPH1 then emerges as a key player in the balancing of light harvesting via regulation of state transitions during the state 2 to state 1 transition. Similar results were published in close connection to Paper II where this protein phosphatase, there referred to as TAP38, was identified as the state transitions phosphatase (Pribil et al. 2010). Interesting to notice from this work is that over-expression of PPH1/TAP38 resulted in faster dephosphorylation of D1 and D2 as well. This indicates that some kind of substrate overlap seems to exist between thylakoid phosphatases similar to what is found for STN7 and STN8 (Bonardi et al. 2005). Pribil et al. also provide data for enhanced growth and photosynthetic performance of the PPH1/TAP38 mutant (Pribil et al. 2010). We did not, however, observe such an improvement of plant fitness in our growth conditions.

Although PPH1 appears to be the LHCII phosphatase involved in dephosphorylation of LHCII subunits and state transitions, there must be another chloroplast phosphatase involved in LHCII dephosphorylation as well. Excessive light inhibits the STN7 kinase and LHCII is known to be rapidly dephosphorylated under such conditions (Rintamäki et al. 1997). This high light-triggered dephosphorylation event of LHCII seems to occur also in the *pph1* mutant. Upon transition from light favouring LHCII phosphorylation to high light, the amount of phosphorylated LHCII is reduced to similar levels in *pph1* and in the wild type (**Figure 9**). Thus, it appears like there are, at least, two phosphatases involved in dephosphorylation of LHCII. PPH1 is clearly responsible for the dark-induced dephosphorylation and for regulation of state transitions. However, the other phosphatase involved in high light-triggered dephosphorylation of LHCII proteins still remains to be identified.

4.2.3. Identification of a mutant deficient in dephosphorylation of PSII core proteins (Paper III)

In the screen for chloroplast phosphatases, a mutant with impaired dephosphorylation of PSII core proteins was recognized and named PBCP (photosystem II core phosphatase). In contrast to the *pph1* mutant, mutant plants lacking PBCP partly retain phosphorylation of the PSII subunits D1, D2 and CP43 upon dark or far-red light

treatment while LHCII phosphorylation is diminished as in the wild type (Figure 1a and S1 in Paper III). Two different mass spectrometric approaches were undertaken to precisely determine which PSII polypeptides were not efficiently dephosphorylated in the mutant. In line with the results from immunoblotting, IMAC experiments revealed significantly higher levels of phosphorylation (approximately two times) for D1 and D2 in *pbcp* compared to wild type after exposure to far-red light (**Figure 11**; Table 1 in Paper III).

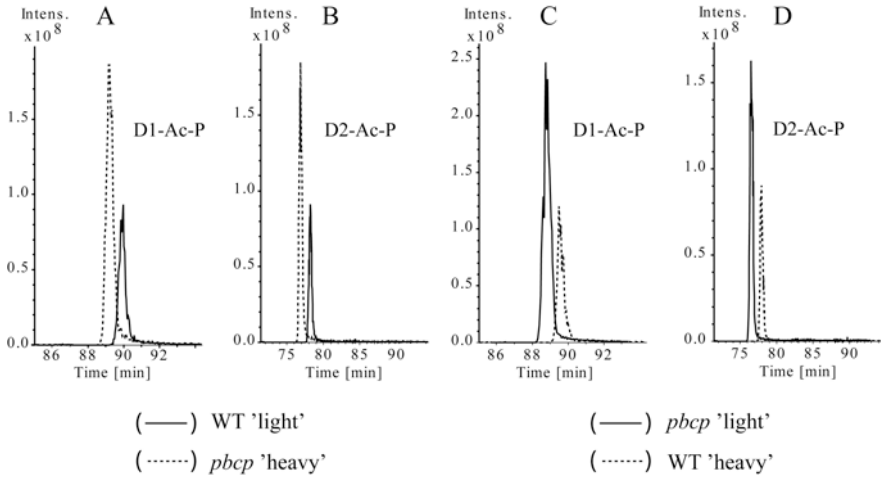

Figure 11. MS analysis of thylakoid protein dephosphorylation using IMAC. (A) and (B) Extracted ion chromatograms of phosphorylated peptides from the *pbcp* mutant labeled with heavy isotope (dashed line) and wild type labeled with light isotope (solid line) analyzed by LC-MS. Peaks corresponding to N-terminal peptides from D1 (A) and D2 (B) are marked. (C) and (D) Extracted ion chromatograms from the reciprocal labeling experiment where peptides from the *pbcp* mutant were labeled with light isotope (solid line) and peptides from wild type were labeled with heavy isotope (dashed line).

In addition, higher levels of phosphorylation of D1 and D2 were also found in mutant plants exposed to blue light when compared with wild type plants although the difference was more pronounced after far-red light treatment. As expected, no significant difference in LHCII phosphorylation was observed after far-red light exposure, as compared to wild type. However, intriguingly, the *pbcp* mutant showed reduced levels of Lhcb1 phosphorylation in blue light. This might be a compensatory effect by higher activities of other phosphatases in the absence of PBCP. The IMAC analysis also showed that the levels of phosphorylated CaS, a STN8 dependent phosphoprotein (Vainonen et al. 2008), were similar in wild type and PBCP-lacking

plants. In addition to the IMAC approach, the difference in PSII phosphorylation was also analyzed by determination of the *in vivo* phosphorylation stoichiometry (% of phosphorylation) for the phosphorylated PSII proteins (Table 2 in Paper III). The result resembled that of the IMAC analysis, but also revealed the involvement of PBCP in dephosphorylation of Thr-2 in PsbH while de-phosphorylation of Thr-4 in PsbH was as efficient in *pbcp* as in the wild type.

4.2.4. Characterization of the pbcp mutant

Like many other phosphatases of the PP2C family, the presence of Mn^{2+} stimulated the activity of recombinant PBCP. Its activity could also be modulated by addition of DTT suggesting a possible redox control mechanism of the enzyme. PBCP is localized to chloroplasts and mainly to the chloroplast stroma (Schliebner et al. 2008; Zybailov et al. 2008; Figure 2B in Paper III). The thylakoid ultrastructure in chloroplasts from the *pbcp* mutant had long regions of few grana membranes with fewer layers compared to wild type (Figure 4 in Paper III). This observation is in direct opposition to what was observed in STN8-deficient plants (Fristedt et al. 2009b). Fristedt et al. found that phosphorylation of PSII subunits controls the macroscopic folding of thylakoid membranes. As PBCP seems to be involved in dephosphorylation of STN8-dependent phosphoproteins, it also seems to affect the folding of thylakoid membranes in an opposite way than STN8.

Since dephosphorylation of PSII proteins has been found to be a central step of D1 turnover during the PSII repair cycle (Ebbert and Godde 1996; Rintamäki et al. 1996; Baena-González et al. 1999), the degradation of D1 during prolonged high light treatment was followed (Figure 5 in Paper III). D1 degradation was clearly retarded in the *pbcp* mutant implicating a role for PBCP in efficient repair of photo-damaged PSII thus being a counterpart to STN8. Although PBCP seems to be specific in dephosphorylation of PSII proteins, over-expression of PBCP results in dephosphorylation of LHCII as well (Figure 6 in Paper III). Similarly, over-expression of PPH1 was found to increase the rate of dephosphorylation for D1 and D2 (Pribil et al. 2010) and an overlap in substrate specificity was also found for the two thylakoid kinases STN7 and STN8 (Bonardi et al. 2005). It is therefore likely that the corresponding phosphatases PPH1 and PBCP have some degree of overlap in substrate specificity. As a consequence of increased LHCII dephosphorylation, the PBCP

overexpressor line was impaired in state transitions. However, the PBCP knockout mutant was not affected in state transitions.

Although PBCP emerges as the phosphatase counterpart to the STN8 kinase, it is, as in the case for PPH1, plausible to think that additional protein phosphatases are involved in dephosphorylation of PSII proteins as well. The strongest evidence for this is the remaining dephosphorylation of PsbH on Thr-4 in the *pbcp* mutant. Also, dephosphorylation of PSII proteins has been found to be dependent on a thylakoid-bound phosphatase whose activity did not depend on the presence of Mn^{2+}. Furthermore, this phosphatase was inhibited by NaF but unaffected in the presence of EDTA which is in clear opposition to what was found for PBCP (Vener et al. 1999). Neither PPH1 nor PBCP are likely to be under regulation of immunophilin CYP38. Taken together, more phosphatases involved in dephosphorylation of thylakoid proteins are expected to be identified.

4.3. Comparative phosphoproteomic analysis of Arabidopsis wild type and mutant plants deficient in STN7 and STN8 (Paper IV)

The finding of PPH1 as the protein phosphatase working in pair with the protein kinase STN7 on LHCII proteins raised the question if PPH1 and STN7 have more substrates in common. Large-scale phosphoproteomic analyses of Arabidopsis identified about 200 phosphorylated chloroplastic proteins distributed between all compartments in this plastid (Sugiyama et al. 2008; Baginsky and Gruissem 2009; Lohrig et al. 2009; Reiland et al. 2009), but little is known about the responsible kinases and phosphatases. In Paper IV we aimed to look for additional substrates of STN7 and STN8 by comparative chloroplast phosphoproteomics. In addition, we also analyzed the *pph1* mutant chloroplast phosphoproteome (unpublished data).

4.3.1. Sample preparation

To make a comparative phosphoproteomic analysis of Arabidopsis wild type and mutant plants I developed a fast isolation procedure of chloroplast proteins. Crude chloroplasts isolated as in (Schubert et al. 2002) were immediately treated with three volumes of acetic acid for rapid inactivation of biological reactions (e.g. kinase and phosphatase activities). $MgCl_2$ was added to a final concentration of 33 mM since acetic acid in

combination with $MgCl_2$ precipitate RNA (Hardy et al. 1969), something that possibly could affect phosphopeptide enrichment. Following trypsin digestion of isolated proteins, phosphopeptides were enriched using TiO_2 affinity chromatography and analysed by mass spectrometry.

4.3.2. Identification of phosphopeptides

Using the described procedure, 27 phosphopeptides from chloroplast proteins were reproducibly identified (**Table 1** and Paper IV). All these peptides have been described earlier (Vener et al. 2001; Reiland et al. 2009; Reiland et al. 2011). As a next step the wild type chloroplast phosphoproteome was compared to that of mutant plants lacking PPH1, STN7, STN8 as well as the double mutant lacking both STN7 and STN8. All phosphorylated peptides found in the wild type were also found in the PPH1 mutant (**Table 1**; unpublished data). The only difference noticed between wild type and PPH1 was the presence of the N-terminal peptide Ac-RKtVAKPK from light harvesting proteins also in samples isolated from dark adapted *pph1* plants, consistent with the findings in Paper II. To find other substrates of this enzyme, a quantitative methodology needs to be undertaken since most proteins identified have some level of phosphorylation both in light and dark conditions. However, the present approach did not allow for such an analysis. Actually, in the present study, only the presence of two peptides was found to be absolutely dependent on the light condition in the wild type: Rubisco activase, that was found only in samples from dark adapted plants, and the above mentioned N-terminal peptide from LHCII.

In contrast to PPH1-deficient plants, the comparative phosphoproteomic analysis revealed several differences between the wild type phosphoproteome and that of plants deficient in STN7 and STN8 (**Table 1**). Two novel findings were obtained: i) neither STN7 nor STN8 kinase was required for the light-independent phosphorylation of Ser-48 in Lhcb1.1-1.3 proteins. ii) phosphorylation of Thr-451 in pTAC16 protein was strictly STN7-dependent.

Interesting to notice is that STN7-dependent phosphorylation of pTAC16 and TSP9 was also found in the dark where STN7 is inactive or at least not phosphorylating LHCII subunits. Similarly, high light-phosphorylation of CP29 has also been reported even though this phosphorylation requires STN7 and the kinase is regarded as inactive under high light conditions (Fristedt and Vener 2011).

Table 1. List of phosphopeptides found in samples from Arabidopsis wild type plants. Phosphopeptides detected (+) or not detected (-) in samples from indicated mutants are labeled in the table. Phosphorylated residues are marked with a lowercase s or t and '*Ac*-' represents N-terminal acetylation.

Protein name	TAIR ID	Phosphopeptide sequence	*pph1*	*stn7*	*stn8*	*stn7stn8*
PsbH	ATCG00710	AtQTVEDSSR	+	+	+	+
		AtQtVEDSSR	+	+	+	+
Lhcb1.1 Lhcb1.2 Lhcb1.3 Lhcb1.5	AT1G29920 AT1G29910 AT1G29930 AT2G34420	*Ac*-RKtVAKPK	+	-	+	-
Lhcb1.1 Lhcb1.2 Lhcb1.3 Lhcb1.4 Lhcb1.5	AT1G29920 AT1G29910 AT1G29930 AT2G34430 AT2G34420	GPSGsPWYGSDR	+	+	+	+
Lhcb4.1	AT5G01530	[NLAGDVIGTRTEAADAK][a]	+	+	+	-
Lhcb4.2	AT3G08940	FGFGtKK	+	-	+	-
		[NLYGEVIGTRTEAVDPK][a]	+	+	+	-
STN7	AT1G68830	tVTETIDEISDGR	+	-	+	-
		tVTEtIDEISDGR	+	-	+	-
D1	ATCG00020	*Ac*-tAILER	+	+	+	-
D2	ATCG00270	*Ac*-tIALGK	+	+	+	+
CP43	ATCG00280	*Ac*-tLFNGTLALAGR	+	+	+	-
TSP9	AT3G47070	EGTTTGGRGtVR	+	-	+	-
		KVDEKEGtTTGGR	+	-	+	-
CaS	AT5G23060	SGtKFLPSSD	+	+	+	-
PsaP	AT2G46820	ATtEVGEAPATTTEAETTELPEIVK	+	+	+	-
		AttEVGEAPATTTEAETTELPEIVK	+	+	+	-
Alb3	AT2G28800	DTVELVEESQSESEEGsDDEEEEAR	+	+	+	-
PsbL	ATCG00560	*Ac*-tQSNPNEQSVELNR	+	+	+	-
Rubisco activase	AT2G39730	GLAYDtSDDQQDITR	+	+	+	+
Unknown	AT3G52230	EATGDDDQKDDDEDDQssDGHED	+	+	+	+
OEP6	AT3G63160	[DKSDSDDATVPPPSGA][a]	+	+	+	+
pTAC16	AT3G46780	VQVAtVR	+	-	+	-
PGRL1A	AT4G22890	[ATTEQSGPVGGDNVDSNVLPYCSINK][a]	+	+	-	-
Unknown	AT4G01150	[ASSEETSSIDTNELITDLK][a]	+	+	-	-
Unknown	AT3G19900	SIVDDEGNLDNsDDEEKESLDDK	+	+	+	+
PsbQ	AT4G05180	FYIQPLsPTEAAAR	+	+	+	+

[a] The exact position of the phosphorylated residue was not determined.

A serine phosphorylation of LHCII that does not require STN7 and is light-independent was surprising. The well-described phosphorylation of LHCII at threonine residues close to the N-terminus is well-studied and known to be important for state transitions. The Thr phosphorylation does not occur in darkness or in the absence of STN7 raising questions about the responsible kinase and function for this STN7- and STN8-independent Ser phosphorylation which is currently absolutely unknown.

4.3.3. Distribution of pTAC16

The finding of pTAC16, a protein found in preparations of transcriptionally active chromosomes (Pfalz et al. 2006), as a STN7 dependent phosphoprotein implicates a possible link between the redox state of the plastoquinone pool and modulation of plastid gene expression. It is known that the redox-regulated kinase STN7 has a role in modulation of plastid and nuclear gene expression but the mechanism by which the kinase affects gene expression is unknown (Pesaresi et al. 2009). Analysis of pTAC16 localization using a pTAC16-specific antibody found it to be distributed between the chloroplast thylakoid membrane and the chloroplast nucleoid (Figure 3 and 4 in Paper IV). Bioinformatics predicts pTAC16 to have a nucleotide binding domain and DNAse treatment of isolated nucleoid indeed resulted in almost complete release of pTAC16 from this fraction. Two other nucleoid-associated proteins, MFP1 and TCP34, believed to anchor DNA to the thylakoid membrane (Jeong et al. 2003; Weber et al. 2006), were found to have a very unusual distribution pattern between nucleoids, proplastids and chloroplasts (Majeran et al. 2012). They were both very abundant in nucleoids and proplastids but their relative concentration in chloroplasts were also high or even higher. The third nucleoid-associated protein that resembled this distribution pattern was pTAC16, suggesting a similar DNA-anchoring role for this protein as well (Majeran et al. 2012).

In order to examine the phosphoproteome of the nucleoid as well as to investigate the distribution of phosphorylated pTAC16 between nucleoids and thylakoids, nucleoid proteins were isolated in the presence of the phosphatase inhibitor NaF. To our surprise, although pTAC16 is an abundant protein in the nucleoid, we were not able to find it phosphorylated within this fraction (Figure 4C in Paper IV). Instead, we found phosphorylated peptides from two other nucleoid-associated proteins, pTAC10 and emb2746, in all biological replicas of the nucleoid fraction. These phosphorylated

proteins are presumably less abundant in the chloroplast compared to pTAC16 since they were only found phosphorylated in one and two biological replicas of the acetic acid extracts, respectively, while pTAC16 phosphorylated at Thr-451 was detected in all biological replicas.

The exclusion of phosphorylated pTAC16 from the nucleoid fraction could imply a regulatory mechanism for the DNA-binding capacity of pTAC16. Similarly, the ability of MFP1 to bind DNA depends on its phosphorylation status (Jeong et al. 2004). Interestingly, STN7, known to be a thylakoid associated protein, is also found within the nucleoid (Majeran et al. 2012) providing a short physical distance between pTAC16 and the kinase. The fact that phosphorylated pTAC16 is excluded from the nucleoids allow me to speculate about a regulatory mechanism in which STN7-mediated phosphorylation of Thr-451 in pTAC16 alters the DNA-binding capacity of pTAC16 leading to modulation of nucleoid functions.

5. SUMMARY OF MAJOR FINDINGS

Before the work on this thesis began, it was suggested that a stable PPIase activity in the thylakoid lumen was an important physiological requirement for plants since FKBP13 was compensating for the loss of activity in the absence of CYP20-2. However, after characterization of mutant plants lacking both FKBP13 and CYP20-2 we now postulate that the functions of immunophilins in the thylakoid lumen of *Arabidopsis thaliana* are not related to their ability to possess PPIase activity and should be investigated beyond this enzymatic activity **(Paper I)**.

Four years ago, the nature of thylakoid protein phosphatases working in pair in with the recently discovered protein kinases STN7 and STN8 was still unknown. After the work of this thesis, we now know that efficient dephosphorylation of LHCII depends on the phosphatase PPH1. PPH1 has a key role in regulation of state transitions **(Paper II)**. In addition, identification and characterization of the protein phosphatase named PBCP revealed a role for this phosphatase in dephosphorylation of PSII subunits, folding of thylakoid membranes and efficient degradation of D1 after photo-damage **(Paper III)**.

Chloroplast gene expression has been found to be affected by the redox-state of plastoquinone and the link between the redox-state of PQ and gene expression is believed to be mediated by a phosphorylation cascade. The finding that phosphorylation of the nucleoid-associated protein pTAC16 requires STN7 provides a possible direct link between the redox-state of photosynthesis and modulation of chloroplast gene expression **(Paper IV)**.

6. CONCLUDING REMARKS

Photosynthetic organisms have evolved during billions of years but our knowledge of how these organisms regulate photosynthesis is far from being complete. The discoveries presented in this thesis add pieces to the puzzle of how plants optimize photosynthesis that eventually will provide us with a comprehensive understanding for these processes. Such knowledge will give us opportunities to improve food production and provide us with the tools to mimic photosynthesis in order to generate renewable energy to meet our increasing need for food and energy supplies.

The Sun must be regarded as the ultimate source of energy that we need to learn how to exploit. Or like James Barber put it: "*After all, there is no shortage of water and the energy content of 1 h of sunlight falling on our planet is equal to all the energy humankind currently uses in 1 year*" (Barber 2006).

7. ACKNOWLEDGEMENTS

First of all, many thanks to all my co-authors without whom this thesis would have been hard to finalize: *Alexey Shapiguzov*, *Thomas Kieselbach*, *Michel Goldschmidt-Clermont*, *Jean-David Rochaix*, *Iga Samol*, *Felix Kessler*, *Charles Andres*, *Geoffrey Fucile* and *Michèle Crèvecoeur*.

Then, thanks to my supervisor Professor *Alexander Vener* for all the help, support and guidance over the years. I admire your exceptional knowledge in, what seems like, everything. I also like many of your expressions associated with science, "*Don't run in front of the locomotive*, *French nouvelle cuisine*, *shy boys will never kiss beautiful girls*, *don't invent the bicycle again*" and, my favourite, "*in my understanding*". The latter is very hard to argue against.

I would also like to thank…
Alexey Shapiguzov for all the help during my first time in the lab, the interesting conversations via gmail and our very fruitful collaborations. I still find it funny that the master and his disciple discovered the same phosphatase independently of each other!

Rikard Fristedt för att ha gjort doktorandtiden extra rolig både på labb och vid våra upptäcktsfärder runt om i världen (de årliga ekoxeexkursionerna i Bjärka-Säby inräknade). Alla dagar på labb med dig har varit grymma! Eller, alla utom en, hehe.

Åsa Jufvas för att du tipsade Alex om en duktig student, allt... hmm... "skvaller", alla roliga grejer vi har gjort på fritiden (+ Öppet Spår ☺) och, framförallt, för att du är en fantastisk vän. Om ett halvår utan dig var så tråkigt som det var, hur trist ska det då inte bli nu?!

Hanna Klang Årstrand för att du har stått ut med mig som kontorskompis samt för alla pratstunder och terapisamtal under åren. Hoppas det blir en varm sommar så att du slipper använda fingervantar inomhus i år!

Maria Turkina för all hjälp med masspektrometrarna och allt skitsnack. Tyst skugga? Verkligen inte!

Cornelia Spetea Wiklund för bra diskussioner och råd under åren som vi var två växtgrupper på fredagsmötena.

Thomas Kieselbach för gott samarbete och nyttiga workshops. Jag är inte rädd för kanariefåglar så har jag vägarna förbi Umeå kommer jag och hälsar på.

Peter Strålfors för hjälp i form av bihandledare, alla roliga anekdoter och trevligt sällskap vid diverse middagar.

Pontus, *Jacob*, *Daniel*, *Pavan* and *Heiner* for bringing new faces and fun into the group for some time during the years.

Lan Yin for the good times at conferences and in the lab. I will always remember the look on your face when the waiter took your plate at the pizza buffet in Copenhagen. You are also my reference to the fact that I'm very good at pipetting!
Patrik Karlsson för trevligt sällskap och konstiga skämt vid våra gemensamma konferenser. "*Men, jag heter ju Milton i andranamn, måste jag dricka igen då?*"
Björn Lundin för att ha gett mig smeknamnet 'Boppe'.
Lorena Ruiz Pavon for all the laughs although it was not easy to have the office in the lab when you were working…

Håkan Wiktander, *Ulf Hannestad*, *Monika Hardmark* och *Camilla Höglund* för att ni fixar alla möjliga typer av problem som uppstår.

All my girls at floor 13; *Anita L*, *Anita S*, *Anna G*, *Anna L-M*, *Annelie*, *Birgitta*, *Chamilly*, *Christine*, *Cilla*, *Emelie*, *Eva*, *Kerstin*, *Lotta*, *Maria*, *Narges*, *Sara*, *Susana*, *Vivian* and *Viviana* as well as the boys *Anders* and *Ingemar*. Thank you for all the fun as well as the never-ending comments about the size of my food portions. Very funny.

Övriga vänner i huset; *Cissi*, *Daniel*, *Elin*, *Elisabeth*, *Ia*, *Jonas*, *Marie*, *Martin*, *Mats*, *Mattias*, *Meenu*, *Pernilla*, *Phiia-Lotta*, *Rikard*, *Sebastian*, *Simon*, *Siri*, *Stefan*, *Sven* och *Tobias* för sällskap i löpar-/skidspåren, kvällar med grillning & vattenskidåkning, trutskådning på soptippen, disputationsfestefterfester med forsknings- & träningsprat till normal frukosttid, hårt motstånd i Boda Borg, handledning under ex-jobb samt trevliga samtal i korridorerna. Dessutom: Förlåt *Sebbe* för att jag dissade dina kakor. De var verkligen fina och... hmm... söta!

Tack även till mina vänner som förgyller livet utanför labbet:

Fenomenet DIPP (*Heta Heto*, *Limmet*, *Mr Press*, *Power*, *Tigern*, *Yxel* och *Zera*) samt dess fans (*Anna-Carin, Annica*, *Emma*, *Flugan*, *Lena*, *Liket* och *Maria*) för tio år av roligheter. Extra tack till *Sebastian Hedlund* vid Max Planck som har skickat över artiklar som jag inte har haft åtkomst till under åren.

Träningsvännerna på Medley; *Emma*, *Fanny*, *Henrik*, *Jörgen*, *Madelene* och *Maria* för allt roligt vi har hittat på i svettiga och osvettiga kläder. Extra tack till min AD *Jörgen Woss* som har hjälpt mig med avhandlingens omslag.

Johan Jufvas och *Pierre Samuelsson* för sällskap (och en hel del motivation...) vid 300km cykling, 90km skidor, 30km löpning och 3km simning.

Tack även till *Roland Ossiansson* och *Ulf Ighe*, mina lärare från högstadiet respektive gymnasiet, som på ett inspirerande sätt intresserade mig för naturvetenskap.

Sist men inte minst vill jag tacka min familj för att ni bryr er om mig och intresserar er för det jag gör även om ni kanske inte alltid förstått vad jag egentligen hållit på med.

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