

Linköping University Medical Dissertations No. 1212

REGULATORY FUNCTIONS OF PROTEIN PHOSPHORYLATION IN PLANT PHOTOSYNTHETIC MEMBRANES

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Linköping 2010

Front / Back cover: Blue native electrophoresis separation of the thylakoid membrane protein complexes with indicated in gel digestions and following mass spectra.

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ISBN 978-91-7393-301-8

ISSN 0345-0082

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Printed by: LiU-Tryck, Linköping 2010.

*Plant a garden in which strange plants grow
and mysteries bloom*

- Ken Kesey

ABSTRACT

Oxygenic photosynthesis is the process in plants, algae and cyanobacteria which converts light energy from the sun into carbohydrates and at the same time produces oxygen from water. Both carbohydrates and oxygen are essential to sustain life on earth. Sunlight is thus a necessity for life, but it can also cause severe problems for photosynthetic organisms, which have evolved several remarkable acclimation systems to cope with light fluctuations in the environment. In higher plants the light driven reactions of photosynthesis proceed in the chloroplast thylakoid membranes highly organized into stacked regions of grana and interconnecting stroma lamellae. The grana structure is thought to provide functional benefits in the processes of acclimation of the photosynthetic apparatus, particularly in the quality control of photosystem II (PSII) where photo-damaged PSII is repaired in a stepwise manner. These processes in the thylakoid membranes were suggested to be regulated by reversible phosphorylation of several proteins in PSII and in its light harvesting antennae complexes (LHCII). Two thylakoid protein kinases, called STN8 and STN7, have been previously identified as responsible for the phosphorylation of PSII and LHCII, respectively. However, molecular mechanisms and the exact functions of these protein phosphorylation events remained largely unknown.

In this thesis research I have demonstrated that the PSII protein phosphorylation is needed for the maintenance of the thylakoid structure in *Arabidopsis thaliana* chloroplasts. A big part of the work on characterization of proteins and their phosphorylation has been done using novel mass spectrometry techniques, and we further developed a label-free method for quantitative studies of protein phosphorylation. The phosphorylation of PSII proteins was found to be diurnal regulated and required for maintenance of the cation-dependent functional stacking of the thylakoid membranes. This phosphorylation was further shown to be important for the regulated turnover of the D1 protein of PSII.

Phosphorylation of the plant specific TSP9 protein was found to be dependent on STN7 kinase, and plants deficient in TSP9 showed reduced ability to perform the photosynthetic state transitions and to execute thermal dissipation of excess light energy under high light conditions. I also accomplished characterization of the protein phosphorylation in thylakoids from *Arabidopsis* plants subjected to high light treatment and discovered STN7-dependent phosphorylation of the

antenna protein CP29 required for the adaptive disassembly of PSII supercomplexes in conditions of high light stress.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Fotosyntes är den biokemiska reaktion där växter, alger och cyanobakterier omvandlar ljusenergi från solen till kolhydrater och samtidigt frigör syre. Syre och kolhydrater är viktiga för allt liv på jorden och solljus är således en nödvändighet för liv men kan samtidigt orsaka allvarliga problem för de fotosyntetiska organismerna. Den biologiska apparaten som styr fotosyntesen är uppbyggd av proteiner som i sin tur är uppbyggda av aminosyror. Proteiner som katalyserar olika specifika biokemiska reaktioner kallas för enzymer, och regleringen av dessa är det som styr cellers förmåga att anpassa sig till sin omgivning. Proteinerna involverade i fotosyntesen är inget undantag, utan dessa regleras på en rad olika sätt för att organismen skall kunna utföra de fotosyntetiska reaktionerna optimalt. Ett sätt för cellen att kontrollera katalytiska proteiner är genom så kallade post-translationella-modifieringar, och en av de viktigaste av dessa är fosforylering. Studier kring fosforylering av olika proteiner i de fotosyntetiska membranen i *Arabidopsis thaliana* (backtrav) under olika ljusförhållanden är stommen i hela denna avhandling. För att på ett kvantitativt och specifikt sätt kunna studera dessa modifieringar har jag bland annat använt en metod som kallas för masspektrometri. Med masspektrometri kan man noggrant väga de aminosyror som proteinerna består av och på så sätt identifiera vilket protein det handlar om. Det är dessutom möjligt att avgöra om detta protein är modifierat.

Växter har utvecklat flera anmärkningsvärda system för att anpassa sig till och hantera olika variationer i den miljö de växer i, detta på grund av att växterna inte kan röra på sig och därmed inte direkt undvika den stress de utsätts för. Ett specifikt särdrag hos högre växter är att deras fotosyntetiska membran (tylakoider) är organiserade i staplade regioner, så kallade grana och de sammanfogande stroma lamella. Denna struktur har visat sig ge funktionella fördelar i de processer som används för dynamiska anpassningar av den fotosyntetiska apparaten. Detta är viktigt i den process som kallas ”fotosystem II kvalitetskontroll” där ljusskadat fotosystem II repareras. Det har länge spekulerats kring hur denna dynamiska aspekt av tylakoiderna regleras genom fosforylering av diverse tylakoid proteiner. Men exakt hur detta sker på molekylär nivå har hittills i stort sett varit höljt i dunkel. De arbetande enzymer som ansvarar för fosforyleringen kallas för kinaser och har identifierats i tylakoiderna som STN7 och STN8 protein kinaser (enzymer). I denna avhandling visar jag att fotosystem II protein fosforylering krävs för underhåll av tylakoidernas struktur i *Arabidopsis thaliana*. Utöver detta visar jag att protein fosforylering

också styr processer som reglerar det komplex som ansvarar för "ljusupptagning" i de fotosyntetiska membranen.

ORIGINAL PUBLICATIONS

- I. **Fristedt R, Willig A, Granath P, Crèvecoeur M, Rochaix JD, Vener AV. (2009)**
Phosphorylation of photosystem II controls functional macroscopic folding of photosynthetic membranes in Arabidopsis. *Plant Cell*. (12):3950-64.

- II. **Fristedt R, Granath P, Vener AV. (2010)**
A protein phosphorylation threshold for functional stacking of plant photosynthetic membranes. *PLoS One*. (6):e10963.

- III. **Fristedt R, Carlberg I, Zygadlo A, Piippo M, Nurmi M, Aro EM, Scheller HV, Vener AV. (2009)**
Intrinsically unstructured phosphoprotein TSP9 regulates light harvesting in *Arabidopsis thaliana*. *Biochemistry*. 48(2):499-509.

- IV. **Fristedt R and Vener AV. (2010)**
High light induced disassembly of photosystem II supercomplexes in *Arabidopsis* requires STN7-dependent phosphorylation of CP29, Manuscript.

OTHER PUBLICATIONS

1. **Moparthy SB, Fristedt R, Mishra R, Almstedt K, Karlsson M, Hammarström P, Carlsson U. (2010)**
Chaperone activity of Cyp18 through hydrophobic condensation that enables rescue of transient misfolded molten globule intermediates. *Biochemistry*. 16;49(6):1137-45.
2. **Sirpiö S, Khrouchtchova A, Allahverdiyeva Y, Hansson M, Fristedt R, Vener AV, Scheller HV, Jensen PE, Haldrup A, Aro EM. (2008)**
AtCYP38 ensures early biogenesis, correct assembly and sustenance of photosystem II. *Plant J*. 55(4):639-51.

ABBREVIATIONS

Ac-	N-terminal acetylation of the protein
CID	collision-induced dissociation
DNA	deoxyribonucleic acid
ESI	electrospray ionization
ETD	electron transfer dissociation
IMAC	immobilized metal affinity chromatography
kDa	kilo Dalton
LC	liquid chromatography
LHC	light harvesting complex
MALDI	matrix-assisted laser desorption ionization
MS	mass spectrometry
MS/MS	tandem mass spectrometry, also called CID or ETD
m/z	mass over charge ratio
NADPH	nicotinamide adenine dinucleotide phosphate
NPQ	non photochemical quenching
OEC	oxygen evolving complex
PSI	photosystem I
PSII	photosystem II
s	phosphorylated serine residue
SDS-PAGE	sodiumdodecylsulphate - polyacrylamide gel electrophoresis
t	phosphorylated threonine residue

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1. Introduction

1.1 Photosynthesis preface

Photosynthetic organisms are able to capture energy from the sun and use it to synthesize organic compounds by means of water and carbon dioxide. Photosynthetic organisms consist of plants, algae and photosynthetic bacteria. Solar energy captured by photosynthesis is used to produce all the biomass on earth and is the ultimate source of energy for many of the living organisms on our planet. Simultaneously these reactions produce oxygen as a side product, also crucially important to most living organisms. Since animals, including humans, cannot directly use the energy from the sun, they need to consume products generated by photosynthesis. The human population that needs such products to survive will soon reach 7,000,000,000. These resources are being used up at a tremendous rate, which gives rise to problems such as global warming, pollution, rainforest felling, war, drought and basically all major issues faced by modern human society. As a result of these problems, and the fact that our very existence depends on photosynthetic reactions, it is essential that we understand how this process works in photosynthetic organisms. By understanding this process, we can ultimately learn to manipulate and control photosynthesis in a way which can help us to enhance the production of food and furthermore make agriculturally important plants more resistant to various environmental conditions. This understanding will lead to higher crop yields to feed the increasing population, or to acquire biofuels.

Another aspect of photosynthetic research is that an understanding of the natural process that has evolved over several billion years can allow us to understand the basic chemistry and physics of photosynthesis. This knowledge can be used to develop solar energy conversion techniques. If solar energy, which is both clean and sustainable, can replace our traditional energy sources such as oil, it would greatly reduce our impact on the global environment. From a holistic perspective, the research mentioned above illustrates the importance of photosynthesis for our planet and its impact on all life on earth.

1.2 Chloroplast and thylakoid membrane structure

Photosynthesis in higher plants and algae is compartmentalized in cell organelles called chloroplasts (Figure 1). Chloroplasts consist of an outer and inner envelope membrane comprising the aqueous protein rich stroma solution and the thylakoid membranes. Carbon fixation takes place in the stroma compartment. The thylakoid membranes have a very distinctive heterogeneous membrane system consisting of appressed regions called the grana, and non-appressed regions connecting these stacks called the stroma lamellae. The components of the packed grana membranes facing towards the stroma are known as grana end membranes, and the turns of each granum are known as margins (Anderson and Andersson, 1988; Albertsson et al., 1990; Albertsson, 2001; Allen and Forsberg, 2001; Mustardy and Garab, 2003). The thylakoid membranes enclose the inner soluble space known as the lumen, which is a compartment that has recently been found to have a complex proteome in itself (Kieselbach et al., 1998). The design of the grana structure has evolved to optimize the photosynthetic process, which allows plants to adapt to fluctuating light conditions (Chow et al., 2005a; Hausler et al., 2009). Grana are found in all higher plants, while most algae and cyanobacteria have very little, or totally lack grana structure (Anderson et al., 2008).

The formation and maintenance of grana in chloroplasts of higher plants is a versatile process controlled by an intricate interplay of several physicochemical forces. These forces are Van Der Waals attraction, entropy, electrostatic attraction and repulsion (phosphorylation), hydration repulsion and steric hindrance (Hodge et al., 1955; Murakami and Packer, 1970; Sculley et al., 1980; Chow et al., 2005b). Furthermore, in the *hcf136* mutant of *Arabidopsis*, which is defective in photosystem II (PSII) assembly, the thylakoid grana is severely enlarged and extended throughout the chloroplast (Meurer et al., 1998). A recent study of chlorophyll b less mutants established that light harvesting complex II (LHCII) complexes also play an important role in the thylakoid structure. This mutant showed less negatively charged and irregularly stacked thylakoids when compared to wild type membranes (Kim et al., 2009). These studies illustrate the importance of protein complex composition in the maintenance of the grana structure.

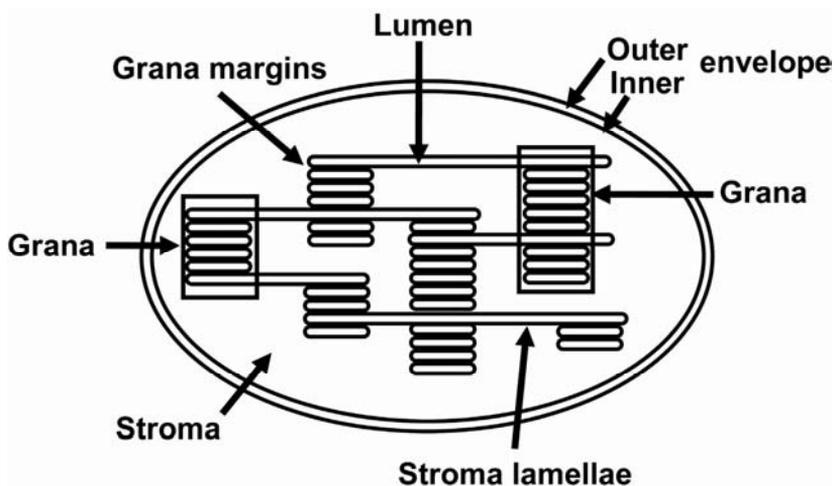


Figure 1. Schematic diagram of the chloroplast. The outer and inner envelope chloroplast membranes enclose the stroma. The photosynthetic thylakoid membrane is divided into the grana stacks and the stroma lamellae, and they enclose a continuous space called the thylakoid lumen.

The molecular organization and uneven distribution of the major photosynthetic complexes within the thylakoid membranes indicates the lateral heterogeneity of these membranes. PSII and LHCII are concentrated in the grana parts while photosystem I (PSI) and the ATP synthase are located in the non-appressed stroma lamellae. The cytochrome b_6f complex (Cyt b_6f) occurs in both membrane types (Andersson and Anderson, 1980; Danielsson et al., 2004). PSII is a membrane protein complex that exists in all oxygenic photosynthetic organisms in which the initial water splitting process occurs (Barber, 2002; Ferreira et al., 2004; Mulo et al., 2008). The core of PSII is composed of the D1 and D2 proteins embedded by CP43 and CP47 inner antennae proteins situated on either side of the D1/D2 reaction center. Also associated to the PSII core is a large number of small intrinsic proteins (Shi and Schroder, 2004). LHCII is the major chlorophyll a/b protein complex and the most abundant protein in the thylakoid membrane in higher plants. LHCII contains three major subunits: Lhcb1, Lhcb2 and Lhcb3. These subunits are present as homo or hetero trimers in combination with PSII (Hankamer et al., 1997). There are also several so-called minor

antenna complexes, referred to as CP24, CP26 and CP29 (Jansson, 1999). In the grana membranes of plants, PSII and LHCII associate to make up the supercomplex which consists of a dimeric core of PSII and two or more LHCII trimers (Dekker and Boekema, 2005; Caffarri et al., 2009).

The functional effects of grana architecture have several important characteristics. These have been demonstrated to depend on protein phosphorylation and size and organization of the thylakoid membranes which are rapidly and dynamically regulated according to different light conditions (Anderson et al., 1973). The area-to-volume ratio of the stacked grana membranes is extremely high and greatly increases light capture which is a result of the massive pigment composition of the interconnectivity of LHCII-PSII supercomplexes (Barber, 1980; Dekker and Boekema, 2005). The spatial separation of the two photosystems also prevents the spillover of energy between PSI and PSII, which is one way to keep the fast (PSI) and slow (PSII) photosystems separate (Barber et al., 1980; Trissl and Wilhelm, 1993). Speculations further imply that the fine-tuning, fast regulatory mechanisms of excitation energy, state transitions and non-photochemical quenching optimize through the grana structure (Horton and Black, 1981; Horton et al., 1996; Lunde et al., 2000; Allen and Forsberg, 2001).

Furthermore, the grana has been shown to be dynamically regulated by changes in both the size of the stacks and the width of the partition gap in response to light conditions (Chow et al., 2005b; Anderson et al., 2008). In connection to this, the regulation of the PSII repair cycle exhibits a dependency on migration of damaged PSII complex between grana and non-appressed membranes (Aro et al., 1993; Aro et al., 2005).

1.3 Photosynthetic energy transformation

The process of oxygen evolving photosynthesis is performed by two photosystems working in sequence, referred to as photosystem I and photosystem II (Allen and Forsberg, 2001). Controlled by these two photosystems, the photosynthetic machinery in the thylakoid membranes converts light into chemical energy in the process generally referred to as the light reactions. Figure 2 illustrates the conversion of solar energy into reducing power in the form of NADPH and ATP (Anderson and Andersson, 1988; Barber, 2002).

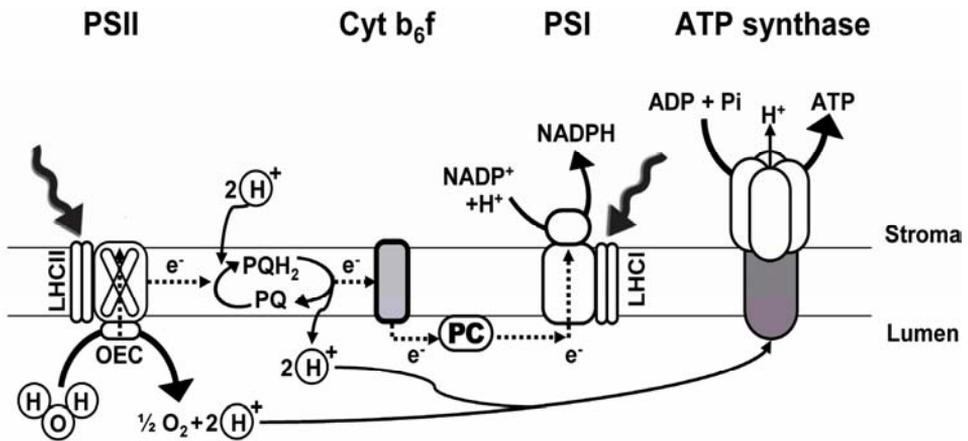


Figure 2. The organization of photosynthetic complexes in the thylakoid membrane and a simplified scheme of the electron and proton transport chain coupled to ATP synthesis.

To produce this reducing power, the energy from sunlight is harvested by the chlorophyll molecules bound to various light harvesting complexes in the thylakoid membranes. The light excitation of chlorophyll molecules induces electron transfer as an initial step. This excitation energy is transferred to the site for charge separation, called the reaction center in PSII, where the primary electron acceptor pheophytin is reduced by accepting an electron. Coinciding with this, the reaction center Chl_a P680 becomes oxidized and loses an electron. On the luminal side of PSII, the oxygen evolving complex (OEC) catalyzes the water splitting reaction and thereby provides a new electron to P680⁺ via a redox active tyrosine residue called Y_z. This process also converts water into oxygen and protons (Nield and Barber, 2006). Electrons migrate from phe to plastoquinone QA, and then to the second quinone acceptor QB. QB accepts two electrons, becomes fully protonated and forms a plastoquinol (PQH₂) which migrates from PSII to the Cyt_b₆f complex to transfer the electrons (Cramer et al., 2005). The small, soluble, copper-containing protein plastocyanin (PC) further transfers the electrons through the lumen to PSI. The electrons from the plastocyanin replace the electrons that have been excited by light absorption in P700 at PSI and the second type of charge separation occurs. On the stromal side of PSI, the electrons are transferred via the

iron-sulfur centers and phylloquinone to ferredoxin (Fd). The final electron transfer from reduced ferredoxin to NADP⁺ is catalyzed by ferredoxin-NADP⁺ reductase. The proton gradient being built up across the thylakoid membrane during the electron transport drives the synthesis of ATP in the ATP synthase complex in a process known as photophosphorylation. The generated energy, as ATP and NADPH, is used in the Calvin-Benson cycle, also referred to as the dark reactions, where the protein complex RuBiSCO fixates CO₂ into carbohydrates (Barber, 2007).

1.4 Thylakoid protein phosphorylation

The phosphorylation of a large number of proteins in the thylakoid membrane complexes is regulated according to environmental conditions (Bennett, 1977). These proteins have been identified as PSII core subunits D1, D2, CP43, PsbH and TSP9, as well as some LHCII polypeptides and the PSI proteins PsaP and PsaD (Hansson and Vener, 2003; Vener, 2007b). Of the minor light harvesting proteins, only CP29 has been found phosphorylated in higher plants (Hansson and Vener, 2003). Recent large scale phosphoproteome analyses have mapped and revealed a complex phosphorylation network in the chloroplasts of *Arabidopsis* (Reiland et al., 2009). The events of protein phosphorylation at the thylakoid membranes are regulated according to the redox state of the protein complexes involved in the electron transport, and so are directly regulated by the light conditions. More specific, distinctive changes in the plant thylakoid protein phosphorylation happen as a response to PSII stress conditions like high light, high temperature or drought stress (Vener et al., 1998; Aro and Ohad, 2003). At the heart of these events, and sensing of the electron transfer chain, are two protein kinases called STN7 and STN8. These enzymes belong to the Ser–Thr kinase family and are conserved in algae and land plants (Depege et al., 2003; Bellafiore et al., 2005; Rochaix, 2007a).

STN8 is involved in phosphorylation of the D1, D2, CP43 and PsbH proteins of PSII, and the calcium-sensing receptor protein Cas (Vainonen et al., 2005; Vainonen et al., 2008). Reversible phosphorylation of D1, D2 and CP43 proteins is suggested to have a function in the repair cycle of PSII, and in particular the prevention of premature degradation of D1 (Aro et al., 1992; Baena-Gonzalez et al., 1999). More specifically, the dephosphorylation of these proteins was shown to coincide with disassembly and migration of PSII in the membrane

from the deep grana towards the stroma parts (Baena-Gonzalez et al., 1999). The highly controlled and organized mechanism of PSII quality control in the chloroplasts of higher plants is connected to the complex heterogenic thylakoid structure in these organelles. These aspects of PSII phosphorylation and thylakoid organization indicate that reversible protein modifications are of significant importance for the dynamic behavior of the protein complexes in the thylakoid membranes. However, the phosphorylation of the PSII proteins is sustained in the dark while being upregulated under light and stress conditions which points to the importance of the PSII phosphorylation being at a certain level during the whole photoperiod (Vener, 2007a). Furthermore, the D1 protein phosphorylation is shown to be dependent on circadian rhythm during the photoperiod (Booij-James et al., 2002). While the phosphorylation of the PSII proteins is always at a certain minimum stable level, the distinct fast dephosphorylation of CP43, D1, D2 is observed when plant leaves are exposed to high temperatures (Rokka et al., 2000).

STN7 is highly specific in the phosphorylation of the light harvesting complex and of the minor light harvesting protein CP29 (Depege et al., 2003; Bellafiore et al., 2005; Tikkanen et al., 2006). The phosphorylation of CP29 in plants is connected to specific conditions such as cold stress and high light (Bergantino et al., 1998; Pursiheimo et al., 2001). Furthermore, as will be revealed in the results section, STN7 has been proven specific in phosphorylation of the soluble TSP9 protein (Fristedt et al., 2009b). STN7 is characterized by having a transmembrane region that separates its catalytic kinase domain in the stroma from the lumen located N-terminus with two conserved cysteine residues. The mechanism of the STN7 kinase regulation is complex with inactivation at increasing light intensity or darkness (Rintamäki et al., 2000). The activation of STN7 is mediated through binding of reduced plastoquinone to the cytochrome b_6/f complex, while the release of the same molecule deactivates STN7 (Vener et al., 1997). STN7 is also regulated at the existence level to ensure that there is a balance between the phosphorylation demand and the amount of STN7 enzyme present (Lemeille S. et al., 2009). The regulation of STN7 is further controlled by the two cysteine residues located in the thylakoid lumen. These amino acids most likely function as targets of reversible oxidation/reduction. It has been demonstrated by site directed mutagenesis that replacement of the cysteines results in both loss of state transitions and LHCII phosphorylation (Rochaix, 2007a).

Furthermore, growing *Arabidopsis* plants under low light conditions and then exposing them to short periods of high light intensity leads to altered expression of stress responsive genes in *stn7* mutant compared to wild type plants (Tikkanen et al., 2006).

The corresponding phosphatase working on LHCII in the de-phosphorylation reaction has recently been characterized. What is intriguing is that these knockout plants have higher biomass production than wild type plants, when grown under low light, although the molecular mechanism for this increase in biomass remains to be determined (Pribil et al., 2010; Shapiguzov et al., 2010). In addition to the LHCII and PSII proteins, subunits of the H⁺-ATP synthase and Rieske Fe-S protein have been shown to become phosphorylated in the thylakoid membranes (Nuhse et al., 2003a; Rinalducci et al., 2006).

1.5 Strategies for molecular adaptation to environmental conditions

The photosynthetic apparatus in the thylakoid membranes of plants is exposed to both seasonal dependent diurnal light cycle and frequent changes in light intensity in the natural environment. These changes in light quality can depend on clouds, shading or sun flecks and therefore change from moment to moment. Longer and more permanent changes in light availability occur on a seasonal basis. The photosynthetic efficiency is optimal at a certain stable light level, but since plants are exposed to an ever changing environment, the ability to regulate both light harvesting and photo-damage are crucial for plant survival (Kruse, 2001).

1.5.1 Non photochemical dissipation of excess excitation energy

The fast reactions used by plants to balance the over-excitation of the photosynthetic machinery involve regulation of light harvesting, which is collectively known as non-photochemical quenching (NPQ) (Baroli and Niyogi, 2000; Horton and Ruban, 2005; Mozzo et al., 2008a; Mozzo et al., 2008b). In this process, the excess excitation energy is released as heat. This suggests that NPQ is the most important photo protective mechanism against high light stress. The kinetics of NPQ work in different time scales and are divided into three kinetic phases of relaxation: the qE rapid phase, qT middle phase, and qI which is the slowest of the three (Lambrev et al., 2010). The quick and reversible phase of qE depends on the proton gradient over the thylakoid membrane, the xanthophyll cycle, and the involvement of the PsbS protein that senses low lumenal pH through two lumen protonable residues

(Demmig-Adams and Adams, 1993; Li et al., 2000; Li et al., 2004; Niyogi et al., 2005). The much slower component qI is associated with photoinhibition of PSII. However, the detailed mechanism of qE is more clearly understood than those of qI and qT.

Recent investigations show that PsbS controls the active state of a membrane protein complex called B4C, composed of the CP29 and CP24 proteins associated with trimeric LHCII. This research demonstrates that the dissociation of this complex is required for the onset of non-photochemical quenching under high light conditions. Mutant plants lacking this complex have strongly reduced heat dissipation abilities (Betterle et al., 2009; van Oort et al., 2010). It was speculated that the minor antenna proteins CP29 and CP24 are involved in a switch mechanism to initiate the energy quenching, and it was further demonstrated that these changes are reversible and thus enable changes in the PSII antenna size as an adaptation mechanism to rapid variations in the environmental conditions (Betterle et al., 2009).

1.5.2 State transitions

Another response to imbalance in available energy is the mechanism of state transitions (Bonaventura and Myers, 1969; Murata, 1969, 2009). This is more of a balancing process under low light conditions that takes place on a time scale of minutes. To optimize for light harvesting, at the photosystem favored under the prevailing light conditions, the LHCII antenna can change association with either PSII or PSI. This mechanism is regulated by the reversible phosphorylation of LHCII, where the non-phosphorylated LHCII (state 1) connects with PSII but as a consequence of phosphorylation moves and associates with PSI (state 2) (Allen et al., 1981; Allen, 1992; Allen and Pfannschmidt, 2000; Allen, 2005; Rochaix, 2007b). The kinase STN7 and LHCII phosphorylation, which occurs because of a reduction of plastoquinone, are essential for state transitions (Vener et al., 1995; Vener et al., 1997; Zito et al., 1999; Haldrup et al., 2001).

1.5.3 Repair of photosystem II

Regardless of the NPQ mechanisms, irreversible damage to PSII occurs and therefore plants have developed an efficient repair process of the damaged proteins (Aro et al., 1993; Tyystjarvi and Aro, 1996; Kanervo et al., 2005). This quality control of PSII involves the turnover of the core PSII subunits and especially the D1 protein; the insertion of a newly

synthesized D1 and the association of cofactors follow this. The chloroplastic ATP-dependent metalloprotease FtsH shows an involvement in the degradation of photo-damaged D1 (Nixon et al., 2005; Komenda et al., 2006; Komenda et al., 2007). Although FtsH is considered as the major D1 protease, it is suggested the thylakoid proteases Deg2 and Deg1 are involved in this process as well (Haussuhl et al., 2001; Pardes et al., 2007). Simultaneously to the D1 degradation, the PSII supercomplex disassembles and the PSII migrates from the appressed grana to the non-appressed stroma lamellae regions. At the stroma lamellae, new D1 copies are synthesized in a light dependent manner and inserted into the PSII complex. When the damaged subunits exchange, the repaired and functional PSII reappear in the grana regions (Aro et al., 1993; van Wijk et al., 1997).

1.5.4 Long term acclimation

The more permanent, long-term acclimation responses to light changes involve the adjustment of photosystem stoichiometry in which the amount of the reaction centers and light-harvesting proteins in the thylakoid membranes adjust in a period of hours and days (Pfannschmidt et al., 1999; Pfannschmidt et al., 2003). Similarly to state transitions, the photosystem stoichiometry changes are regulated by the redox state of the plastoquinone (Allen and Pfannschmidt, 2000; Brautigam et al., 2009; Steiner et al., 2009). Recent investigations have revealed an involvement of the STN7 kinase in the long-term adaptation process as well (Pesaresi et al., 2009).

2. Methodologies

2.1 *Arabidopsis thaliana* as a model organism

Arabidopsis thaliana is a small flowering plant of the mustard family (Brassicaceae). The genome of *Arabidopsis* was sequenced more than 10 years ago and has since become the most widely used model organism for plant cell biology and genetics research (The Arabidopsis Genome Initiative, 2000). The advantage of using *Arabidopsis* is the small genome, short generation time and the ease of collecting large amounts of seeds. The plants used in this work were grown in a temperature controlled climate room using a hydroponic setup (Norén et al., 2004) with an 8h light photoperiod at $120 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ at 20°C . For high light treatment, $800\text{-}900 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ light was used.

The insertion of T-DNA at the gene of interest can be used to study the corresponding characteristics of the mutant, which lacks a certain protein compared to wild type plants. I used this powerful method as a large part of my work, and the detailed characterization of the homozygous *tsp9*, *stn7*, *stn8* and *stn7stn8* mutants are described in the papers I and III.

2.2 Acrylamide gel electrophoresis

Gel electrophoresis is one of the primely methods for protein analysis. Native PAGE (Poly Acrylamide Gel Electrophoresis) separates proteins under native conditions. SDS-PAGE is an example of a denaturing electrophoresis technique. Both of these methods were used to electrophoretically transfer the proteins in the gel to a blotting membrane to make immunological analysis with several different antibodies. These methods are both described in more detail in the corresponding papers. To study protein phosphorylation, both phosphothreonine antibodies from New England Biolabs and ZYMED Laboratories were used. Furthermore, a direct quantitative gel staining approach using ProQ diamond phosphoprotein staining and the complementary SYPRO Ruby total protein stain (Molecular Probes) was used.

2.3 Mass spectrometry and proteomics

Proteins are the building blocks of life, and to determine the different properties of individual proteins is one of the most important tasks of modern biology research. The large scale analysis of proteins and their posttranslational modifications by mass spectrometry can be defined as analytical protein chemistry or proteomics (Mann et al., 2001). The study of a proteome is much more complex than a genomic study because the genome of a cell is more or less constant while the proteome is much larger than the genome, due to alternative splicing and post-translational modifications like phosphorylation (Mann et al., 2001; Mann and Jensen, 2003). Furthermore, the dynamic range of different protein concentrations can be in many orders of magnitude, thus complicating the study of low abundant proteins (Aebersold, 2003).

These hurdles have resulted in the development of suitable techniques which make it possible to perform a detailed protein analysis. Mass spectrometry is one of the most favorable methods used to study large scale protein dynamics today. The development of the ionization techniques used to charge proteins (Electrospray ionization and Matrix-assisted laser desorption ionization) was awarded the Nobel Prize in chemistry 2002 with one half going to John B. Fenn and Koichi Tanaka (Tanaka et al., 1988; Fenn et al., 1989) for the development of these methods. The MALDI ionization creates ions by excitation with a laser and generates singly charged ions and MALDI is normally used in conjugation with a TOF (Time of Flight) analyzer. ESI, in contrast to MALDI, generates multiple charged ions that can be used to make tandem mass spectrometry (MS/MS) in combination with various mass filters to obtain structural amino acid information for the peptides of interest. The flourishing technology of mass spectrometry development in recent years has resulted in extremely sensitive techniques capable of accurate protein identifications. By using reverse – phase high performance liquid chromatography, it is possible to separate the peptides according to their reversible interaction with the hydrophobic surface of the stationary phase of the chromatographic medium before the ESI-MS analysis. Furthermore, by using nanoliter-LC coupled to tandem mass spectrometry (nano-LC-MS/MS), excellent sensitivity and separation for the quantitative analysis of phosphorylated peptides has been achieved (Jensen, 2004; Trelle et al., 2009).

The proteomic strategy used in this thesis employ a method were thylakoid membranes are incubated with trypsin to release the parts of the proteins protruding out of the membranes, including the phosphorylated domains (Vener and Stralfors, 2005; Turkina et al., 2006; Turkina and Vener, 2007). This method, termed vectorial proteomics, greatly reduces the complexity of the sample; however in many cases it is essential to employ enrichment of the phosphopeptides prior to MS analyses. By using the high affinity of the phosphate groups to Fe (III) ions on IMAC columns the phosphopeptides can be enriched. To further increase their specificity methyl estrification of the peptides prior to loading onto the IMAC column was used (Nuhse et al., 2003b; Vainonen et al., 2005). To measure the dynamics of thylakoid protein phosphorylation and to be able to make comparative studies under different light conditions I used a method involving stable isotopes, as well as an approach utilizing label-free comparisons of peptides (Steen et al., 2005), and their corresponding modified versions. Figure 3 shows the main steps for a typical proteomics approach used in this research.

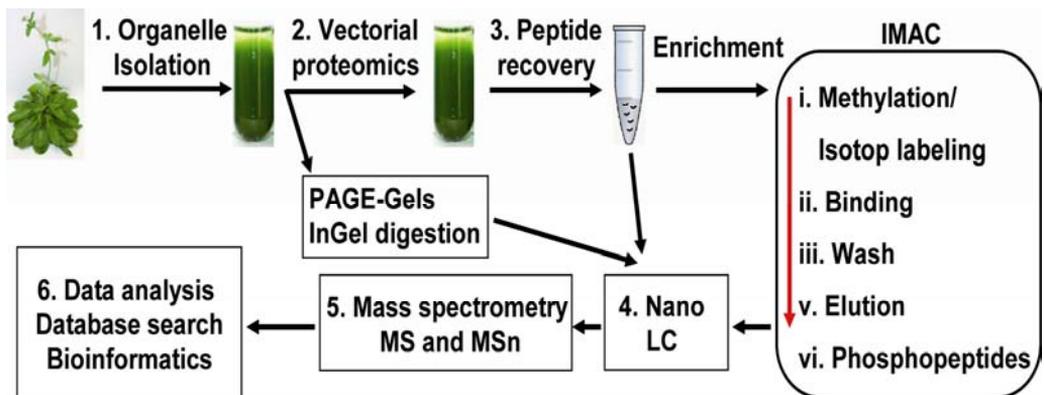


Figure 3. Workflow of proteomic analysis of the thylakoid membranes used in this work. Thylakoids were isolated from *Arabidopsis* leaves. These membranes were analysed using PAGE gels. Alternatively, the surface protruding parts of the thylakoid proteins were released by trypsin, and then were sequenced directly using MS/MS. For phosphopeptide purification the peptides were subjected to IMAC and the eluted phosphopeptides were identified with MS/MS (MSn).

2.4 Chlorophyll fluorescence measurements

The fluorescence emission kinetics was measured using both a portable Chlorophyll Fluorometer PAM200 and a stationary pulse amplitude modulation 101-103 fluorometer (Walz, Effeltrich, Germany).

2.5 Thylakoid membranes

If the membrane organization of the thylakoids is greatly altered between mutant and wild type plants this can be observed in faster (or slower) sedimentation of these membranes in transparent PVC tubes. I developed a method termed “Gravity-driven sedimentation” where I photographed the sedimentation of isolated membranes during periodic time intervals.

For subfractionation of thylakoids into grana and stroma membrane parts, a digitonin solution of 2% was used and the mixture was homogenized in a glass homogenizer five times and mixed for 5 min at room temp. The rest of the procedure is described in (Fristedt et al., 2009a).

3. Present Investigation

3.1 Aims

In recent years there have been some controversial and ambiguous interpretations of the results concerning the exact role of protein phosphorylation in the thylakoid membranes of higher plant chloroplasts. And although many of the mechanisms have been elucidated, the exact role of these post-translational events partly remains unclear. With respect to this, the general aim of this research work has been to elucidate the exact role and functional details of protein phosphorylation in the photosynthetic membranes.

The more specific aims in the projects are:

Paper I: To investigate the role of PSII core protein phosphorylation.

Paper II: Quantitative measurements of the PSII phosphorylation stoichiometry, and to investigate the functional cation-dependent stacking of plant thylakoid membranes.

Paper III: The functional characterization of the small thylakoid protein known as the thylakoid soluble phosphoprotein of 9kDa (TSP9).

Paper IV: To identify the high light regulated phosphorylation response in the thylakoid membranes - with specific focus on the minor antenna protein CP29.

3.2 PSII phosphorylation and the dynamic structure of the thylakoid membrane

3.2.1 Remaining phosphorylation in the *stn7stn8* plants

The conformational change in the structure of LHCII and the change in the lateral organization of the thylakoid membrane upon phosphorylation has been determined (Varkonyi et al., 2009). Although the functional phosphorylation of the PSII core proteins is greatly investigated, the exact role of these events and especially the connection between D1 turnover and PSII phosphorylation is controversial and the current results are conflicting (Bonardi et al., 2005; Tikkanena M. et al., 2008). To investigate this complex question, I conducted a detailed characterization of the dynamic thylakoid properties in the kinase mutant lacking the most of the thylakoid phosphorylation (*stn7stn8*). This mutant lacks both thylakoid associated protein kinases STN7 and STN8 described above. Comparative western blotting, using phosphothreonine antibodies on membranes from wild type and *stn7stn8*, revealed remaining low amounts of phosphorylation in *stn7stn8* plants (Figure 4). However, the analysis of the phosphorylation situation in *stn7stn8* has previously been examined using immunodetection with antiphosphothreonine antibodies and it was then concluded that *stn7stn8* totally lacks any phosphorylation of the PSII proteins. To determine the precise phosphorylation status in the thylakoid membranes of the *stn7stn8* plants I used an MS approach.

Two phosphorylated peptides were detected from *stn7stn8* and the phosphorylation sites were precisely characterized to: Thr-1 phosphorylated N terminus of the D2 protein and Thr-2 phosphorylated N terminus of the PsbH protein. As shown in Figure 4 this is also visualized with immunoblotting using phosphothreonine antibodies. It was quite surprising to find remaining phosphorylation in the membranes from the *stn7stn8* plants since this mutant was previously found to totally lack phosphorylation. Nevertheless, it is interesting to note that in a recent study by Blomqvist et al. only the PSII proteins PsbH and D2 were found in prolamellar bodies and prothylakoids, suggesting an involvement of these proteins in the early development of the thylakoid membranes (Blomqvist, 2009). At that early stage of thylakoid structure formation there may be a so far unidentified kinase phosphorylating D2 and PsbH to prevent the early grana from collapsing. However, the role for the

phosphorylation remaining at these proteins is currently not known, and the corresponding kinase/s is/are yet to be identified.

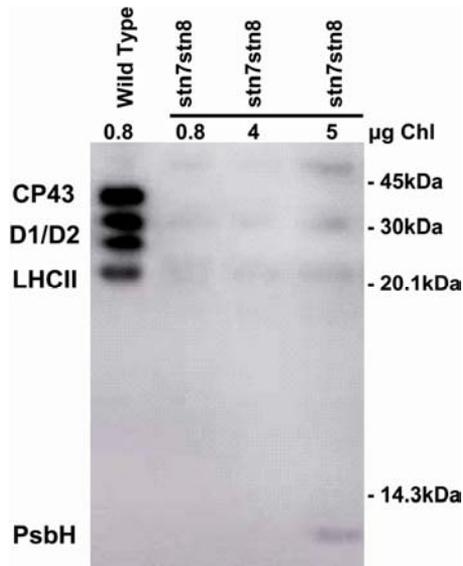


Figure 4. PSII protein phosphorylation visualized by Western blotting. Thylakoid membranes from both wild type plants and *stn7stn8* mutant were isolated and separated by SDS-PAGE. Western blotting was performed using phosphothreonine antibodies from New England Biolabs. The amount of Chl loaded is indicated above each lane: for wild type 0.8µg Chl and for *stn7stn8* 0.8, 4 and 5µg Chl. Low molecular weight markers are indicated to the right of the blot.

3.2.2 PSII protein phosphorylation regulates thylakoid folding

One of the first things I noticed when started to work with the membranes from *stn7stn8* was the strange feature of faster sedimentation of the thylakoid preparation compared to wild type thylakoid suspension. To clarify these observations thylakoid membranes from wild type and *stn7stn8* plants were incubated side by side in transparent PVC tubes and photographed at certain time intervals. As visualized in Figure 5, there is a clear increase in the rate of gravity driven sedimentation observed for thylakoids isolated

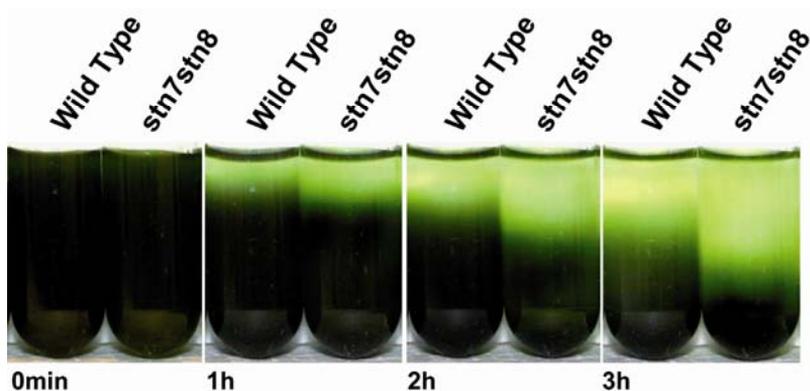


Figure 5. Gravity driven sedimentation of thylakoid membranes from the wild type and *stn7stn8* leaves displayed at different time points. The thylakoid membranes were isolated from plants exposed to normal light ($120 \mu\text{mol photons m}^{-2} \text{s}^{-1}$).

from *stn7stn8*. While not knowing the reason for faster sedimentation in *stn7stn8*, our first guess was that these membranes had increased folding and consequently, density. To investigate this, and directly visualize the membranes, transmission electron microscopy was used.

These observations revealed a striking difference in grana folding between *stn7stn8* and wild type membranes (Figure 6). The results from these observations elucidated an important consequence of the phosphorylation in the thylakoid membranes, namely its involvement in the dynamic structure of these biological membrane systems. The impact of reversible protein phosphorylation on the macroscopic thylakoid structure is a novel finding, and since this structure should be regulated according to the prevailing environmental conditions I conducted a quantitative method to study this further. The phosphorylation of the PSII proteins was first studied using phosphothreonine antibodies and thylakoid membranes were prepared from plants at different time points during the photoperiod. It is clearly evident from Figure 7 that the phosphorylation of PSII was accurately controlled for the various PSII proteins during the photoperiod, although in different directions. By using HPLC in conjunction with Electro Spray Ionization Mass Spectrometry it was possible to resolve the

major phosphorylated peptides and their non-phosphorylated counterparts and to determine the phosphorylation stoichiometry for PSII proteins (Table I).

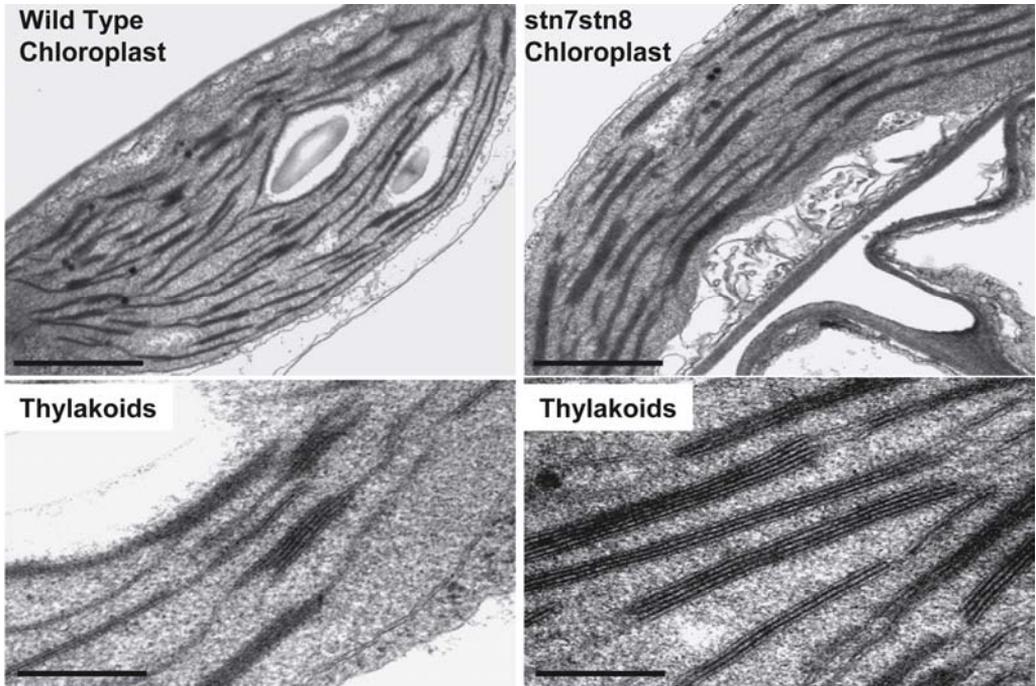


Figure 6. Chloroplasts and thylakoid membranes from the wild type and *stn7stn8* plants visualized by electron microscopy. Chloroplast and thylakoid sections are shown for the wild type (left panels) and *stn7stn8* (right panels). Bars in the top and the bottom panels correspond to 1 and 0.5 μ m, respectively.

The results show that the average phosphorylation state of the wild type PSII core proteins D1, D2, CP43 and PsbH never decreases below \sim 30%. Furthermore, the phosphorylation state is regulated according to the dark/light cycle so that the CP43 phosphorylation is up-regulated during the dark hours which is opposite to the D1 and D2 core proteins of PSII and LHCII (Figure 7, Table I). This opposite regulation of the phosphorylation state is very interesting and could be important for the stable structure of plant thylakoids.

Table I. In vivo phosphorylation stoichiometry (% of phosphorylation) for the PSII core proteins from dark, normal light and high light treated wild type leaves.

	Ligh conditions:		
	Dark	Normal	High
Protein:	% Phosphorylation %		
P-CP43	49±12	34±13	41±13
P-D1	27±12	36±15	55±12
P-D2	33±11	42±12	67±11
P-PsbH	29±9	24±12	34±10
PP-PsbH	N.D.	54±14	63±16

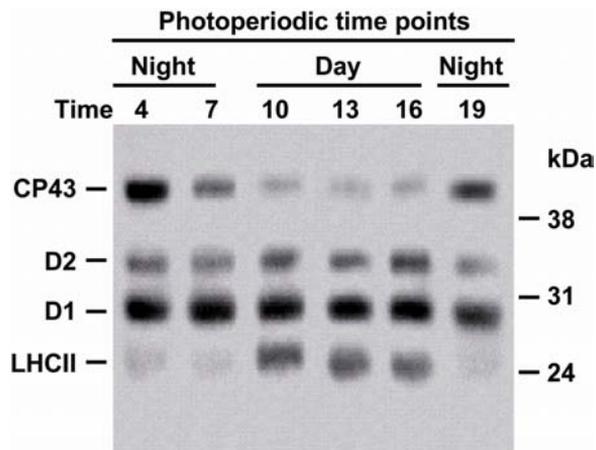


Figure 7. Diurnal changes in the PSII and LHCII protein phosphorylation detected by anti-phosphothreonine antibody. Thylakoids were prepared from wild type plants harvested at the indicated time points during night (4, 7, 19) or day (10, 13, 16) of the photoperiod.

This means that the thylakoid structure is controlled by the level of PSII phosphorylation, and the phosphorylation of PSII proteins is regulated to be maintained at a certain steady state level throughout the photoperiod. Furthermore, if the dynamic ability of the thylakoid membranes is controlled by the PSII phosphorylation there should also be a connection to high light, since under these conditions the demand for flexibility is high. I extended the investigation and also did quantitation of the PSII phosphorylation under high light, and as can be seen in Table I there is a clear increase in PSII phosphorylation during high light treatment.

3.2.3 Kinases regulating the light dependent PSII protein phosphorylation

To clarify and investigate the involvement of the STN7 and STN8 kinases in PSII phosphorylation during dark or light conditions, the mass spectrometry based quantitative approach was extended to involve the *stn7*, *stn8* and *stn7stn8* mutants as well (Table II). The phosphorylation dynamics of the PSII core proteins is dependent on the STN8 kinase (Table II). The number of phosphoryl groups per PSII in wild type or *stn7* was very similar with ~ 1.3 during night and ~ 2.5 during day. In the *stn8* plants, on the other hand, the number was ~ 0.5 during the night and 0.47 during the day (Paper II).

Table II. In vivo phosphorylation stoichiometry (% of phosphorylation) for the PSII core proteins from wild type, *stn7*, *stn8* and *stn7stn8* mutant plants harvested during day or night. N.D. – not determined. *The PsbH and D2 phosphopeptide signals were detected in the samples from *stn7stn8* only in one or two out of three different experiments.

	Wild Type		<i>stn8</i>	
	Dark	Light	Dark	Light
Protein	% Phosphorylation %		% Phosphorylation %	
P-CP43	49±12	34±13	14±8	13±7
P-D1	27±12	36±15	12±9	14±8
P-D2	33±11	42±12	10±6	10±4
P-PsbH	29±9	24±12	11±8	9±8
PP-PsbH	N.D.	54±14	N.D.	N.D.
	<i>stn7</i>		<i>stn7stn8</i>	
	Dark	Light	Dark	Light
Protein				
P-CP43	44±13	36±9	N.D.	N.D.
P-D1	29±12	35±8	N.D.	N.D.
P-D2	33±13	44±7	<3*	<4*
P-PsbH	29±15	22±8	<4*	<4*
PP-PsbH	N.D.	57±9	N.D.	N.D.

3.2.4 The grana structure controls the D1 Turnover rate

Previously, it was reported that the D1 turnover in the repair cycle of PSII was slower in plants lacking the STN8 kinase (Tikkanen et al., 2008). In paper I, I show that this impairment of D1 turnover is a consequence of the missing PSII phosphorylation, that is to say; less dynamic flexibility of the thylakoid membranes in plants lacking the STN8 kinase. One of the proteases mainly involved in D1 turnover in plants is an ATP dependent zinc metalloprotease called FtsH (Nixon et al., 2005). The FtsH is a membrane protease complex consisting of six large subunits protruding into the stroma; this probably makes FtsH largely excluded from the tightly appressed grana membranes. The turnover of D1 is the core of the PSII repair cycle and takes place in all photosynthetic organisms (Yokthongwattana and Melis, 2006). However, the rate of the D1 turnover is much slower in higher plants as compared to cyanobacteria as a consequence of the more structured thylakoid membrane in higher plants chloroplasts. Due to this structure, the maintenance of PSII in the thylakoids of higher plants involves the movement of PSII between the stacked grana membranes toward the unstacked stroma lamellae where replacement and insertion of a newly synthesized D1 can take place. It is probable that the slower turnover of D1 in *stn7stn8* is a consequence of the increased grana size which ultimately leads to the decreased dynamic mobility of the proteins involved in the turnover of PSII (illustrated in Figure 8). Indeed, I found that FtsH was excluded from the grana membranes of *stn7stn8* plants, this illustrates the core function of the PSII phosphorylation, namely to allow for dynamic distribution of the protein complexes in the thylakoid compartments under the repair and quality control of PSII.

This phosphorylation dependent distribution of protein complexes probably works in multiple directions, besides optimizing size of the grana. Firstly, for FtsH, this means that this huge complex can move into the grana under conditions of massive phosphorylation. Secondly, there can be forces of repulsion and attraction between different protein complexes controlling the movement and associations of these complexes according to phosphorylation (addition of negative charge).

To add to the dimension of regulation of these processes is the dependence of thylakoid structure of divalent cations like Mg^{++} . The negative charge added to the

membranes by phosphorylation is balanced by saturation with the Mg^{++} ions, thus creating an optimal state between the repulsing forces of the phosphogroups at the opposing PSII complexes in the grana (Figure 8). However, how this regulation is controlled and if certain ion transporters are involved in the regulation of the Mg^{++} concentration in the soluble stroma is currently unknown.

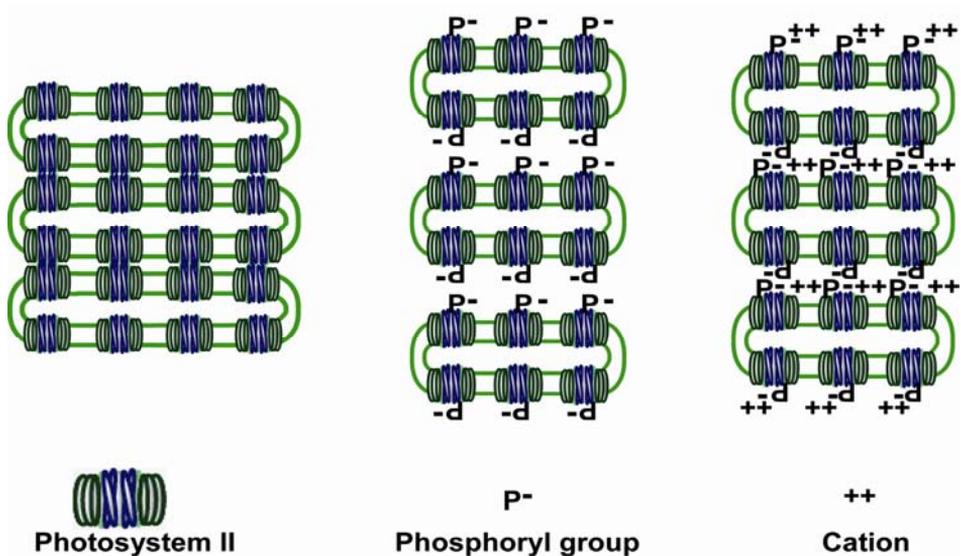


Figure 8. The model showing phosphorylation and cation-dependent dynamic regulation of plant thylakoid membrane structure. Deficiency in protein phosphorylation of photosystem II proteins (*stn8* and *stn7stn8* mutant plants) causes enlargement of grana (left). The presence of at least one phosphorylgroup per photosystem II in wild type *Arabidopsis thaliana* thylakoids causes electrostatic repulsion of the adjacent grana membranes (center). The phosphorylation of the wild type membranes makes their stacking highly cation-dependent (right).

3.3 Regulation of light harvesting is adjusted by protein phosphorylation

3.3.1 The intrinsically unstructured phosphoprotein TSP9

The molecular details regarding the process of state transitions are constantly being refined and the classical view of this mechanism has recently been updated (Tikkanen et al., 2006). This process is much more distinct in green algae compared to higher plants chloroplasts. To investigate the regulation of state transitions in plants, attention need to be focused on differences between these two photosynthetic organisms that basically exert the same process to balance the light harvesting efficiency but to different extends, and also possibly with different functions. TSP9 is an plant specific thylakoid phosphoprotein with a mass of 9 kDa, with unknown function but known to be soluble. It is released from the thylakoid membranes upon light treatment and subsequent phosphorylation, and furthermore it makes associations with both photosystems and the light harvesting complex (Carlberg et al., 2003; Hansson et al., 2007). Genes for homologous proteins were found in *Arabidopsis* and 48 other plant species but not from any other organisms, making TSP9 a plant specific protein. Furthermore, TSP9 showed the interesting behavior to acquire certain structural features when binding to membrane mimetic micelles, as was revealed by NMR spectroscopy (Song, 2006). The primary goal of Paper III was to study the function of TSP9 in *Arabidopsis*, especially since the already known properties of this protein suggest its involvement in the regulation of light harvesting. By using the available microarray data in the Genevestigator database (<https://www.genevestigator.ethz.ch/at/>), the expression of TSP9 showed to be specific to the green photosynthetic tissues and also to be differentially expressed under different light conditions (Paper III).

3.3.2 STN7 kinase is involved in light-controlled phosphorylation of TSP9

Recombinant TSP9, incubated together with thylakoid membranes from wild type or the kinase mutants lacking STN8 kinase showed increased phosphorylation of TSP9 upon illumination, while the membranes from *stn7* plants failed to phosphorylate TSP9 (Figure 9). Since the STN7 kinase is required for state transitions and for the phosphorylation of LHCII

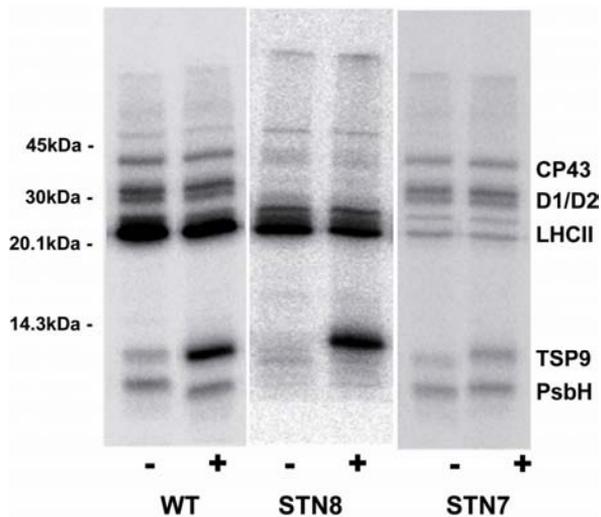


Figure 9. Phosphorylation of recombinant TSP9 protein. Thylakoid membranes from wild type (WT) *stn8*, and *stn7* *Arabidopsis* plants were illuminated in the presence of [γ - 32 P]ATP with (+) or without (-) addition of recombinant TSP9 protein.

and CP29, this finding was very important and the involvement of TSP9 in regulation of LHCII phosphorylation was further demonstrated by fluorescence measurements and western blotting (Paper III). These results clearly show that the plants lacking TSP9 have a decreased ability to execute the state transition mechanism. However, although the STN7 mutant totally lacks the ability to perform state transitions, the TSP9 mutant was not affected to the same extent. This suggests an involvement of TSP9 as a regulatory unit of LHCII, either as a

mediator between STN7 and LHCII or directly as a linker between LHCII and PSII. Furthermore, these investigations demonstrate yet another dimension when the TSP9 lacking plants exhibit a decreased capacity to perform NPQ at high light intensity (Paper III). The rapidly forming, and reversible, qE part of NPQ has been studied in great detail and has been shown to be regulated by the thylakoid Δ pH gradient and the PsbS protein. With this in mind, it is interesting that TSP9 has been found to interact with the PsbS protein (Hansson et al., 2007).

3.3.3 TSP9 is involved in the stability of the PSII-LHCII supercomplexes

Even though the state transition and the NPQ are two different regulatory responses, the involvement of TSP9 in these reactions should be based on the same regulatory basis. The answer to this question came from investigations on the stability of the different protein complexes in the thylakoid membranes of the *tsp9* mutant. The PSII-LHCII supercomplexes were shown to have increased stability in the plants lacking TSP9. Since structural changes and dissociation of LHCII from the supercomplex are the fundamental basis for both the state transition and NPQ mechanisms, the increase in stability of the supercomplexes in the mutant could be the reason to why these processes are impaired in the *tsp9* mutant (Paper III). The structural analysis of TSP9 put this protein in a family of intrinsically unstructured proteins containing long regions lacking fixed structure. These proteins are found in 33% of eukaryotic proteins and are associated with signal transduction, cellular regulation, and assembly of large macromolecular complexes (Tompa, 2005). To investigate the possible involvement of TSP9 in light induced gene regulation, analyzes of the difference in gene expression between the *tsp9* mutant and wild-type plants using micro array was performed. A specific set of mostly signaling or unknown proteins that were not upregulated in high light in the mutant lacking TSP9 was found. However, these proteins did not overlap with those found affected in plants lacking STN7 in accordance with the other recent publication (Pesaresi et al., 2009).

In plants an important feature to sustain the changing environmental conditions is the ability to dynamically regulate certain units of the photosynthetic apparatus. The properties of the TSP9 protein and the behavior of the TSP9 knockout plants certainly place this protein in the pool of these regulatory units.

3.3.4 High light induced phosphorylation of CP29 protein is required for disassembly of photosystem II supercomplexes

The involvement of the TSP9 protein in the dissipation of excess energy upon high light stress, and the fact that LHCII phosphorylation is affected in the *tsp9* mutant (Paper III), indicates phosphorylation dependent regulation of NPQ. While the function of PSII and LHCII phosphorylation is becoming clear in the PSII quality control and the state transition mechanism, the function of minor antenna CP29 phosphorylation has so far remained unclear. CP29 phosphorylation is the only one of the light harvesting proteins that requires strong reduction of the plastoquinone pool (Bassi R et al., 1997; Mauro et al., 1997). Furthermore, hyperphosphorylation of CP29 under high light has been suggested to work as an uncoupler between LHCII and PSII in *Chlamydomonas reinhardtii* (Turkina et al., 2006). It was also recently demonstrated that reorganizations in the grana membranes is involved in NPQ regulation through the PsbS protein. Under high light conditions, a complex consisting of CP29, CP24 and LHCII dissociated from the PSII supercomplex and migrated to the periphery of the grana. It was suggested that this movement of CP29 and CP24 associated these proteins with PsbS as a part of the short term energy dissipation mechanism (Betterle et al., 2009).

I characterized the differential association of CP29 with thylakoid protein complexes under normal and high light conditions. Blue native gel separation was performed on the major thylakoid protein complexes from both normal and high light treated plants (Figure 10). However, numerous repetitions of the Blue native gels did not reveal any significant difference in the protein complexes pattern between normal and high light. By using a quantitative mass spectrometry method on the excised protein complexes from the blue native gel, I found that the spots containing PSII complexes also consist of huge amounts of PSI proteins (Paper IV). This would indicate that it can be problematic to visualize differences in protein complex composition between samples, since the PSI proteins can mask PSII proteins and vice versa. To circumvent this problem I used immunoblotting with specific antibodies on the Blue native gels. The high light treatment led to an obvious relocation of CP29 from the PSII supercomplex to PSII di- and monomers (Figure 10). In addition to the Blue native

analysis, I also conducted a phosphoproteomics approach to the thylakoid membranes and the multiple phosphorylation of CP29 was found to be high light regulated (Paper IV).

3.3.5 High light regulated CP29 phosphorylation depends on STN7 kinase

In connection with these results, it was recently demonstrated that *stn7* mutants displayed a retarded phenotype when growing in fluctuating normal and high light conditions (Tikkanen et al., 2010). I conducted the same experiments on *stn7* mutant plants as done for wild type under normal and high light conditions (Figure 10). The *stn7* mutant showed a remarkable difference compared to wild type, namely that CP29 remained attached to the supercomplexes under high light (Figure 10). Also, it was shown that *stn7* clearly is needed for the proper phosphorylation of CP29 (Paper IV).

The results concluded from Paper IV show that the high light induced STN7-dependent phosphorylation of CP29 is needed for a proper dissociation of PSII-LHCII supercomplexes. This dissociation is most likely a mechanism to regulate the dissipation of excess excitation energy, and is controlled by phosphorylation.

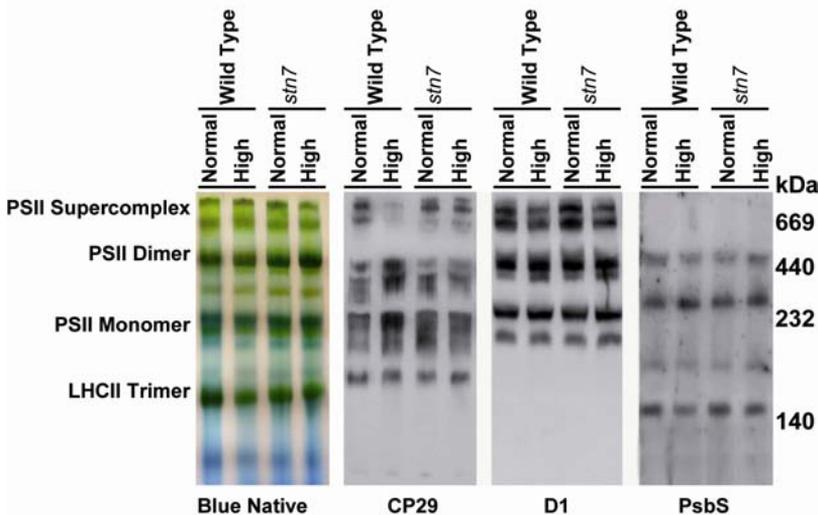


Figure 10. Blue native electrophoresis separation of the thylakoid membrane complexes from normal and high light treated wild type and *stn7* mutant plants and subsequent immunodetection. Representative blue native gel showing different types of PSII complexes is shown in the left panel. Western blots of similar gels with CP29, D1 and PsbS antibodies are shown in the other panels, as indicated.

4. Conclusions

I made characterization of the dynamic phosphorylation of photosystem II and light harvesting complex II proteins in connection to the adaptation responses of photosynthesis in *Arabidopsis thaliana*. The summary of the most important conclusions drawn from the results of the research described in this thesis are:

- Reversible phosphorylation of photosystem II proteins controls the macroscopic structure of the photosynthetic thylakoid membranes. This structural organization impacts the lateral mobility of proteins required for repair and sustained activity of photosystem II.
- The phosphorylation of four photosystem II core proteins is regulated in different directions according to the light/dark cycle, probably to sustain the grana structure.
- Phosphorylation of photosystem II proteins is required for regulation of the thylakoid membrane structure by the cations like Mg^{++} .
- TSP9 phosphorylation is regulated by STN7 and TSP9 is found to be involved in initiation of both state transitions and dissipation of heat. TSP9 probably facilitates the dissociation of light harvesting complex II from photosystem II, which is an important step in the regulation of light harvesting. Furthermore, a specific set of proteins expresses differently between wild type and *tsp9* plants under high light.
- STN7 lacking plants have a decreased mobility of the CP29 antenna protein under high light conditions. This demonstrates that STN7 is involved in the high light regulated unpacking of the PSII-LHCII supercomplexes, which is most likely a mechanism to initiate both photosystem II quality control and heat dissipation.

5. Concluding remarks

The major conclusions of this thesis are that thylakoid protein phosphorylation has evolved as a genius way to tackle the many different environmental challenges that higher plant chloroplast endure. These dynamic processes put the two kinases STN7 and STN8 in the center of this regulation. Although an impressive amount of research exists on these enzymes, we do not fully understand many things.

In the future, we will probably fully understand and be able to control these posttranslational protein processes. This might be a way to control certain plant characteristics and to develop crops with specific properties. Increased production of biomass through improved agronomic performance will ensure generations of sustainable energy resources in the future.

6. Acknowledgments

First of all a general massive thanks to all my co-workers, friends and family!

Especially I would like to thank...

My supervisor Professor *Alexander Vener* for excellent guidance through the world of biochemistry and mass spectrometry. Your outstanding knowledge in science and everything surrounding it is very inspiring and I am truly thankful for your generosity and support during the past years. In my eyes you are a perfect supervisor and pedagogue!

Inger Carlberg for nice collaborations, your kindness and words of advice was really great in the beginning to get things going.

Professor *Cornelia Spetea Wiklund* for many important discussions and thoughtful comments.

Professor *Uno Carlsson* for providing a fantastic atmosphere for me to learn biochemistry from the beginning, and also for letting me do my master thesis in your lab.

Maria Ahnlund my mentor during my first time in the lab, you learned me so much!

Professor *Peter Strålfors* for always being so helpful and nice.

Annelie Lindström, thank you so much for putting up with all my trouble at the end!

Professor *Peter Söderkvist* and *Johan Edqvist* for support during my half time seminar.

The group friends (past and present):

Maria Turkina a great officemate, I will miss the interesting and fun talks about everything from antics to mass spectrometry.

Björn Ingelsson the best lab friend there can be! It is really nice to have someone like you around! BBE.

Pontus Granath big thanks to you for teaming up with me in the laboratory during a hectic time. BBFE.

Åsa Jufvas the best traveling friend and fun company. BTOE.

Alexey Shapiguzov my former officemate, I really miss you in the lab!

Hanna Årstrand Klang really nice to have you back. BTE.

Jacob Kuruvilla one of the coolest and nicest persons I have meet.

Björn Lundin thank you for all the fun we had at conferences and in the lab.

Lorena Ruiz Pavon I miss your nice laugh and your positive energy!

Anna Edvardsson, Georgious Mercapouotilous, Sophie Heurtel, for being so friendly!

Lan Yin and *Patrik Karlsson* for the great traveling company to conferences and also for all the important scientific discussions during our Friday meetings.

Ulf Hannestad, Håkan Wiktander for just being outstanding problem solvers!

Jason, the grammar master!

Thanks to all the super nice people at IKE Cellbiology. *Sven, Mats, Risul, Annelie, Anette, Åsa, Jonas, Lena, Deepti, Lotta, Cilla, Maria, Johan, Chamilly, Tobias, Vivian, Eva, Elisavet, Anna, Emelie, Anita, Kerstin, Ingmar, Anders, Tobias, Sebastian, Cissi, Siri.*

The homeboys, *Jolle, Adde, Edvin, Fredde, Eka, Sam S, Shurebrothers band* - heavy tunes!

Thanks for all the positive energy and the so needed relaxation times away from science!

My mother *Gunilla, Olle och Karin, Nesim, Wilgot*, you are all very special to me.

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